TRYPANOSOMA (TRYPANOZOON) EVANSI (STEEL, 1885): IMMUNE RESPONSES AND IMMUNOSUPPRESSION DURING EXPERIMENTAL INFECTION IN SHEEP

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DEDICATION

To my wife, Nkechinyere Gloria and our daughters, Chiamaka Nnennaya and Solumkenechukwu Iheoma.

Your support and sacrifices are priceless.

Your enduring love is the power.

DECLARATION

I hereby declare that the work presented in this thesis was carried out entirely by myself except where specifically stated in the text and in the acknowledgements

Denis N. Onah

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ABBREVIATIONS

α	-	Alpha
AIDS	-	Acquired immunedeficiency syndrome
APC	-	Antigen presenting cell
APCs	-	Antigen presenting cells
β	-	Beta
BCG	-	Bacille Calmette Guerin
BDV	-	Border disease virus
BNMS	-	Biotinylated normal mouse serum
BSA	-	Bovine serum albumin
B.Vet.C	-	British Veterinary codex
^{o}C	-	Degrees centigrade
CBPP	-	Contagious bovine pleuropneumonia
CD	-	Cluster of differentiation
CFA	-	Complete Freund's adjuvant
CMI	-	Cell mediated immunity
Con A	-	Concanavalin A
CPA	-	Cyclophosphamide
CPM	-	Counts per minute
CTVM	-	Centre for Tropical Veterinary Medicine
DASh	-	Donkey anti-sheep
δ	-	Delta
DEAE-52(DE-52)	-	Diethylaminoethyl cellulose
DMSO	-	Dimethyl Sulphoxide
DN	-	Double negative
DNA	-	Deoxyribonucleic acid
DNFB	-	Dinitrofluorobenzene
DNP	-	Dintrophenol
DP	-	Double positive
DTH	-	Delayed type hypersensitivity
EDTA	-	Ethylenediamine tetracetate
ELC	-	Expression-linked copy
ELISA	-	Enzyme-linked immunosorbent assay
EM	-	Electron microscopy
Fab_2	-	Antigen binding fraction of an antibody molecule
FACS	-	Fluorescence activated cell sorter
Fc	-	Crystallizable fraction of an antibody molecule responsible for binding to antibody receptors on cells and the C1q component of complement.
FCS	-	Fetal Calf Serum
FITC	-	Fluorescein isothiocyanate
FL	-	Fluorescence
FL1	-	Green fluorescence
FL2	-	Red fluorescence
FMD	-	Foot-and-mouth disease
FMDV	-	Foot-and-mouth disease virus
FSC	-	Forward angle light scatter
γ	-	Gamma
GAM	-	Goat anti-mouse
GAR	-	Goat anti-rabbit
GM-CSF	-	Granulocyte macrophage-colony stimulating factor
H-2	-	Mouse major histocompatibility complex
HCT	-	Haematocrit centrifugation technique
HIV	-	Human immunodeficiency virus
HRP	-	Horseradish peroxidase
^{3}H-TdR	-	Tritiated methylhydrogen thymidine

IEL	-	Intraepithelial lymphocytes
IFB	-	Immunofluorescence buffer
IFN-γ	-	Gamma interferon
Ig	-	Immunoglobulin
IgA	-	Immunoglobulin A
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
IL-1	-	Interleukin 1
IL-2	-	Interleukin 2
IL-2R	-	Interleukin 2 receptor
IL-3	-	Interleukin 3
IL-4	-	Interleukin 4
IL-5	-	Interleukin 5
IL-6	-	Interleukin 6
IL-10	-	Interleukin 10
IPP	-	Ileal Peyer's patches
i.v.	-	intravenous
kD	-	kilodalton
Kg	-	kilogram
KLH	-	Keyhole Limpet Haemocyanin
LCA	-	Leucocyte common antigen
LM	-	Light microscopy
LPS	-	Lipopolysaccharide
LT	-	Lymphotoxin
mAb	-	Monoclonal antibody
mAbs	-	Monoclonal antibodies
Mel Cy	-	Cymerlarsan
mg	-	Milligram
MHC	-	Major histocompatibility complex
ml	-	Millilitre
MLC	-	Mixed lymphocyte culture
MLR	-	Mixed lymphocyte reaction
Mls	-	Minor lymphocyte stimulation loci
mm	-	Millimeter
mMol	-	Millimolar
mRNA	-	Messenger ribonucleic acid
mVAT	-	Metacyclic variable antigen type
MW	-	Molecular weight
μ	-	Microns
μCi	-	Microcurrie
μg	-	Microgram
μl	-	Microlitre
μm	-	Micrometer
^{o}N	-	Degrees North
nm	-	Nanometer
NMS	-	Normal mouse serum
O.D.	-	Optical Density
OVA	-	Ovalbumin
P.ag	-	*Pasteruella* antigen
p.b.	-	Post booster
PBL	-	Peripheral blood leucocyte
PBLs	-	Peripheral blood leucocytes
PS	-	Phosphate saline
PBMC	-	Peripheral blood mononuclear cell
PBS	-	Phosphate buffered saline
PCD	-	Programmed cell death
PCV	-	Packed cell volume
PE-SA	-	Phycoerythrin-streptavidin complex
PFC	-	Plaque forming cells

PG	-	Prostaglandin
PGE1	-	Prostaglandin E1
PGE2	-	Prostaglandin E2
PHA	-	Phytohaemagglutinin
p.i.	-	Post infection
P.P.D.	-	Purified protein derivative of tuberculin
P.P.P.	-	Prepatent period
PSG	-	Phosphate buffered saline glucose
p.v.	-	Post vaccination
PWM	-	Pokeweed mitogen
RAS	-	Rat anti-sheep
RF	-	Rheumatoid factor
r.p.m.	-	Rotations per minute
RSV	-	Respiratory Syncytial virus
S-19	-	Strain 19
SBU	-	Sheep biology unit
SDS-PAGE	-	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGOT	-	Serum glutamate oxaloacetate transaminase
SGPT	-	Serum glutamate pyruvate transaminase
ShAM	-	Sheep anti-mouse
S.I.	-	Stimulation index
sIg	-	Surface immunoglobulin
SPF	-	Specific Pathogen free
SRBC	-	Sheep red blood cells
SSC	-	Side angle light scatter
SSE	-	Sodium Salicylate extract
T.ag	-	Trypanosomal antigen
TcR	-	T cell receptor
TfR	-	Transferrin receptor
T_H	-	T helper cells
TM	-	Trade mark
TMB	-	Tetramethyl benzidine
TREU	-	Trypanosome Research Edinburgh University
TWBC	-	Total white blood cell counts
VAT	-	Variable antigen type
VPM	-	Veterinary Pathology, Moredun
VSG	-	Variant specific glycoprotein
WBC	-	White blood cells

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ABSTRACT OF THESIS (Regulation 3.5.10)

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This thesis describes studies on immune responses and immunosuppression during experimental *Trypanosoma evansi* infections in sheep. The studies were initiated by investigations into: 1) routine parasitology and haematology, 2) cell population dynamics in blood and, 3) assessment of parasite-specific antibody responses. Investigations into the role of *T. evansi* in suppression of immune responses to heterologous antigens were carried out using *Pasteurella haemolytica* vaccine as a model antigen and involved: 1) assessment of local inflammation, and measurement of skin thickness, at the site of vaccination, 2) cell phenotype dynamics in blood and in efferent lymph draining the site of vaccination, 3) assay of anti-*Pasteurella* antibodies, 4) cellular responses to T and B cell mitogens, *Pasteurella* and homologous trypanosomal antigens *in vitro* and, 5) cell depletion experiments to try and identify the cell phenotype(s) responsible for immunosuppression.

T. evansi infections in sheep produced scanty and frequently cryptic parasitaemia, ending in selfcure in some cases. There was marked lymphocytic leucocytosis from day 22 post infection (p.i.). Indirect immunofluorescence and flow cytometry revealed decreases in the proportions of $CD5^+$, $CD4^+$, $CD8^+$ and SBU-$T19^+$ ($\gamma\delta$) T cells, which were paralleled by increases in the sIg^+, $CD45R^+$, $CD1^+$ cells and cells expressing the MHC Class II antigen. These changes were relatively minor in the selfcured sheep except that there was a greater decrease in $CD8^+$ than $CD4^+$ cells resulting in increased CD4:CD8 ratio. Trypanosome-specific IgG and IgM antibodies were detected in all infected animals by enzyme immunoassays. However, the levels of these antibodies were greater in the selfcured animals.

T. evansi infection actively suppressed the local inflammation and skin thickness normally induced in uninfected control sheep by subcutaneous inoculation of *Pasteurella* vaccine. Moreover, early systemic mobilisation of neutrophils as seen in uninfected controls was suppressed. Analysis of cell dynamics in the blood of infected, vaccinated sheep revealed that vaccination did not reverse the changes in the proportions of T cell subsets and B cells which was induced by *T. evansi* in the infected sheep. Two-colour flow cytometry also showed that *T. evansi* induced increases of up to 70 percent in B cells expressing the CD5 antigen ($CD5^+$ B cells) which persisted after vaccination but was minimal in the selfcured animals. Vaccination caused little alteration in the normal expression of these lymphocyte subsets in the uninfected control sheep. In efferent lymph draining vaccination sites there were no major changes in the proportion of the T cell subsets in either group of sheep. However, two-colour flow cytometry revealed that infection was accompanied by the appearance of $CD4^+CD8^+$ (double positive, DP) cells. These increased in proportion upon vaccination, with up to 30 and 60 percent of $CD4^+$ and $CD8^+$ cells respectively being DP. Moreover, between 40 to 90 percent of B cells were $CD5^+$. In contrast, in uninfected control sheep, the changes resulting in the co-expression of various T cell subsets and CD5 antigen on B cells were observed to a very limited extent. Anti-*Pasteurella* antibodies of the isotypes IgG1, IgG2 and IgM were produced in all immunised sheep, including those infected with *T. evansi*. However, infection of sheep prior to vaccination resulted in the suppression of IgG antibody responses and production of non-specific IgM antibodies in that substantial level of this antibody was detected in the serum collected from these animals prior to vaccine administrations. In contrast, the selfcured sheep responded to secondary vaccination and produced IgG antibodies at levels similar to those seen in vaccinated, uninfected control sheep.

In vitro cell culture systems revealed that the proliferative responses of lymphocytes from infected sheep were depressed when stimulated with T or B cell mitogens, Concanavalin A (Con A) or bacterial lipopolysaccharide (LPS) respectively. Active infection also suppressed the responses of cells to specific stimulation with *Pasteurella* and homologous trypanosomal antigens. Treatment with trypanocidal drug restored these proliferative responses. These suppressive effects were not observed with cells collected from uninfected control sheep, even when 5×10^5 trypanosomes ml^{-1} or ultrasonicated trypanosomal antigens were added to the cultures. Cell depletion prior to culture showed that although $CD8^+$ T cells and cells of the monocyte/macrophage lineage play a significant role in the suppression of lymphoproliferative responses, the latter cell type seems to play the more predominant role. Removal of either of these cell types restored the responsiveness of the cells to mitogenic or specific antigenic stimulus to varying degrees. The responses were enhanced further by simultaneous removal of both cell types from the cell population.

CHAPTER ONE

General Introduction

Trypanosomes are obligate haemoflagellate parasites which occur in all classes of the phylum vertebrata. However, it is in mammals that they assume immense veterinary and medical importance. Most species of pathogenic trypanosomes are carried by haematophagous dipteran flies of the genus *Glossina* which inhabit well over 10 million km^2 of Africa (FAO/WHO, 1969; Kershaw, 1970; Jordan, 1986).

Trypanosoma rhodesiense and *T. gambiense* are responsible for trypanosomosis in humans, causing East and West African sleeping sickness respectively. *T. brucei*, *T. congolense* and *T. vivax* are the causative agents of the livestock trypanosomoses commonly known as nagana. These species, in partnership with their arthropod vector have been responsible for the deaths of tens of thousands of people and hundreds of thousands of their domesticated livestock. In addition, they have played a prime role in limiting the pace and extent of rural development in much of tropical Africa (Nash, 1969; Jordan, 1986).

In contrast, *T. evansi*, the aetiological agent of the disease known as surra, while also affecting a wide range of domestic animal hosts is, unlike the nagana species, more widely distributed as a result of its adaptation to the mechanical mode of transmission, occurring in countries in South America and South East Asia (Hoare, 1972; Stephen, 1986; Urquhart and Holmes, 1987). *T. vivax* has also spread beyond the tsetse infested areas of Africa to the West Indies, Central and South America and Mauritius (Hoare, 1972). The spread is also facilitated by the ease with which *T. vivax*, despite its biological transmission by *Glossina*, adapts itself to mechanical vectors. This adaptation is probably determined by its primitive cycle of development in *Glossina*.

Although trypanosomes and the disease they cause have been known for over 100 years and despite the fact that much effort has gone into attempts at controlling them, nothing much has changed. Thus, in Africa, trypanosomoses still deny vast areas of land to all domestic livestock except poultry and it is estimated that 45 million people, 147 million cattle, 125 million goats and over 103 million sheep remain under threat of the disease (Hornby, 1952; FAO/WHO/OIE, 1982; UNDP/WORLD BANK/WHO, 1983; Dwinger, 1985). In South America and South East Asia where the mechanically transmitted trypanosomoses occur, there is a vast livestock population (Table 1.1). Although trypanosomosis is widespread in these areas the actual number of animals at risk is not known. However, what little data is available indicates that the possible scale of disease is substantial. For example, in Indonesia with a buffalo, cattle and horse population of 3.4, 10.6 and 0.74 million respectively (FAO/WHO/OIE, 1990), the number of surra cases diagnosed increased from a total of 14,000 in 1983 to 28,000 in 1984 (Anonymous, 1985: cited by Payne

et al., 1991). Similarly, in Argentina with a horse population of 3 million (FAO, 1989; FAO/WHO/OIE, 1990) and where *T. evansi* parasitosis is widely distributed, surra constitutes a disease of great economic importance (Monzon and Mancebo, 1986) with a prevalence rate of 20 percent (Monzon and Villavicencio, 1990).

Table 1.1 **Estimates of livestock populations in the surra zones of South America and South East Asia (based on FAO, 1989; FAO/WHO/OIE, 1982; 1990). All figures are in millions.**

	Buffaloes	Camels	Cattle	Goats	Sheep	Horses
South America	1.2	15	292	84	439	54
South East Asia	441	3	1200	826	889	25

The term 'trypanosomosis' [plural = trypanosomoses] (Kassai *et al.*, 1988) describes a complex of allied diseases, each of which is due to infection with one species or several species of pathogenic trypanosome(s). In tsetse-transmitted trypanosomosis, the trypanosomes, after ingestion by the fly in the blood from an infected host, undergo a cycle of development in the fly's digestive tract which terminates in the production of infective forms termed metacyclic trypanosomes. Infection is transferred to a new and susceptible host by inoculative means when it is fed upon by an infected fly. There are characteristic cycles of biological development in *Glossina* which depend on the species of trypanosomes. For example, in the Salivarian trypanosomes, development commences in the fly midgut and terminates in the salivary gland for the *brucei* group (*T. brucei brucei*, *T.b. gambiense* and *T.b. rhodesiense*), and in the proboscis for *T. congolense*, *T. simiae* and *T. suis*. The entire cycle of development of *T. uniforme* and *T. vivax* is restricted to the proboscis. On the other hand, the cycle of development of the Stercorarian trypanosomes e.g. *T. cruzi*, commences in the midgut of the vector and terminates in the faecal medium of the hindgut. In contrast, *T. evansi* has lost its capacity for cyclical development in arthropod vectors and is transmitted mechanically from one susceptible host to another during interrupted blood meals by haematophagous flies other than *Glossina*. Principal among several of such mechanical disseminators of *T. evansi* are the 'Horse' or 'Breeze' fly, *Tabanus* and the 'stable' fly, *Stomoxys*.

Over the years, considerable effort has gone into tsetse and trypanosomosis research in the hope of finding effective means of controlling and eradicating the disease. This is rationalised by the concept that tsetse and

trypanosomosis eradication will directly improve the protein-deficient diets of the majority of the African rural population through improved livestock production. In consequence, various radical trypanosomosis control measures, some of which later resulted in massive land degradation and environmental pollution, were instituted in various parts of Africa (Jordan, 1986). Such measures fall into three broad categories namely: those directed against the parasites, those directed against the vectors (*Glossina*) and those centred on the hosts.

Measures directed against the parasites include chemotherapy and chemoprophylaxis. Chemotherapy is the most widely employed and perhaps the most successful approach to trypanosomosis control. In many countries it is the only and most sensible approach because in the short term, it is cheaper and easier to kill the parasite than it is to attempt to kill the vector (Jordan, 1986). However, in the long term, chemotherapy can become very expensive (Dwinger, 1985) and does not have any permanent effect on the cycle of the disease. On the contrary, if treatment is relaxed, the incidence of disease could soon return to pretreatment levels due to the existence of animal reservoirs of the parasite (Jordan, 1986). Moreover, in many countries, the extent of current reliance on drugs for the control of animal trypanosomosis is alarming. Since only a limited number of drugs is available and their use often abused due to inadequate veterinary supervision, drug resistant strains of trypanosomes have evolved in many areas. Chemoprophylaxis, an alternative to chemotherapy has been employed in several countries where trypanosomosis occurs. To be successful however, it requires even stricter supervision than chemotherapy. If it is to work, a prophylactic drug must be administered and readministered before the level of the first dose drops to a critical level below which the trypanosome can survive, multiply and possibly develop resistance. Such a regime requires the presence of trained staff, reliable transport and easy access to the animals involved (Leach and Roberts, 1981). Chemoprophylaxis is thus inappropriate for use with nomadic or semi-nomadic livestock in pastoral systems of management but can be successfully employed with stock maintained on well organised ranches (Putt *et al.*, 1980; Jordan, 1986).

Measures directed against the vectors (principally *Glossina*) include (a) the destruction of game (reservoir) animals upon which they feed, (b) the clearing of vegetation and destruction of tsetse breeding habitats, (c) the application of insecticides, (d) the use of traps and insect-impregnated targets and (e) the breeding and release of sterile male tsetse flies to compete with wild males. In recent years, most tsetse control and eradication campaigns have been effected almost exclusively by aerial application of insecticides. Opinions differ as to the relative advantages of this measure over those directed against the parasite, given the environmental

consequences. A major attraction of the campaigns to stop the transmission of trypanosomosis by elimination of *Glossina spp* is the economic advantage of eradication over control. Despite the economic advantage of tsetse eradication, in comparison with a commitment to repeated long-term control measures, this radical solution is only feasible over a relatively small proportion of the area of Africa infected by tsetse flies (Jordan, 1986). This notwithstanding, tsetse eradication campaigns have in the past been executed with a reasonable degree of success in Zululand (Du Toit, 1954), on the Island of Principe (De Azevedo *et al.*, 1962), over extensive areas of Northern Nigeria (Davies, 1964; 1971; MacLennan and Na'isa, 1971; Nigeria, 1973-75; 1976-77; Spielberger *et al.*, 1977; Jordan, 1986), in Zimbabwe (Chapman, 1976) and in Botswana (Davies and Bowles, 1979).

Control measures involving the host include avoiding or restricting the movement of animals into areas of risk, movement of animals out of endemic areas and breeding of animals for trypanotolerance. There is potential in the use of breeds which are resistant to trypanosomosis such as the Mutura, N'dama and other West African cattle breeds as well as East and West African indigenous sheep and goats (Griffin and Allonby, 1979; ILCA, 1979). Trypanotolerant animals are known to survive infections without trypanocidal drug intervention and remain as productive as other breeds (Roberts and Gray, 1973; ILCA, 1979, Murray *et al.*, 1981). Moreover, trypanotolerant N'dama and Muturu cattle are also less susceptible to other diseases such as anaplasmosis, babesiosis and heartwater (Epstein, 1971), streptothricosis (Coleman, 1967) and helminthosis (Murray, Morrison and Whitelaw, 1982). However, the population of trypanotolerant animals is low, with a restricted distribution. For them to have an impact on the control of trypanosomosis there needs to be a large increase in their numbers and distribution. Given that these animals are often kept as status symbols, it will require radical sociological changes as well as enormous financial commitment to multiply and introduce these trypanotolerant livestock into the vast areas presently at risk from trypanosomoses.

T. evansi, which occurs in countries of Africa, the Americas, Asia, parts of Europe, the middle and far Eastern countries (Hoare, 1972; Stephen, 1986) is the least researched of all pathogenic trypanosomes despite being the first pathogenic mammalian trypanosome species to be discovered. This fact is probably a result of its much argued phylogenetic releationship with *T. brucei* (Leese, 1911; 1927) and the assumption that information obtained from *T. brucei* studies could be applicable to *T. evansi*. In a series of papers, later collated in 1972, Hoare provided strong and impressive arguments in support of this phylogenetic relation. However, the loss by *T. evansi* of the ability of cyclical development in *Glossina* and its adaptation exclusively to mechanical means of transmission have imposed basic, albeit

important biological differences between the two, negating direct extrapolation of results derived from the study of one to the other, irrespective of phylogenetic relationships. For instance, although *T. brucei* loses its pleomorphism and the ability to develop cyclically in *Glossina* following serial syringe passage in experimental animals there has been no evidence of sustained natural maintenance and transmission of the parasite in the absence of *Glossina*. In addition, cyclical development in the vector engenders certain morphological, ultrastructural and biochemical changes in the parasite. These changes do not occur in *T. evansi* since its multiplication is restricted to the vertebrate host. Moreover, the adaptation to exclusive mechanical transmission has facilitated an extremely wide distribution of *T. evansi* among a wide range of hosts, and although Tabanid flies are its main transmitters, several other haematophagous arthropods as well as vampire bats are known to transmit it (Leese, 1909; Nieschulz, 1930; Dunn, 1932). This fact further presents basic epizootiological differences between *T. brucei* and *T. evansi* which negates the application of similar control strategies. For instance, whereas control programmes designed to eliminate *Glossina* could have radical impact on curbing the maintenance and transmission of *T. brucei*, such measures may have little or no impact on the control of *T. evansi* whose mechanical vectors possess different biology and patterns of life cycles from *Glossina*.

It is generally accepted that *T. evansi* does not cause serious disease in cattle, goats, sheep, pigs and buffaloes, although this is not in fact the case. Its most severe manifestation of disease is produced in horses and camels. This may explain why so little attention has been paid in the recent past to this parasite by research scientists and veterinarians since there is limited interest in horses and camels compared with other livestock. Nevertheless, it is often overlooked that trypanosomes in general produce their most far-reaching effect through their restrictions on the keeping of livestock used for draught power. This in turn has limited the development of farming systems utilising animal traction and the availability of farmyard manure for boosting foodcrop production. The use of donkeys, mules, cattle, buffaloes and horses for draught purposes offers the best possible means for increased food crop production to farmers in most third world countries. They can greatly increase the area that can be cleared and cultivated by one man at much less expense than by any mechanized means (Connor, 1989). *T. evansi* is known to have played a significant role in limiting the use and performance of transport and draught animals in countries of the Mediterranean littoral, Northern regions of West Africa, Euroasia, India as well as islands of the Indian and Pacific oceans (Adiwinata and Dachlan, 1969). Such animals, even though the infections are supposedly mild and chronic, often succumb as a result of the added

stress of work and frequent marginal planes of nutrition. Hence, mortalities of 60, 90 and 100 percent have been reported in goats, buffaloes and horses respectively (Rodenwaldt and Doewes, 1921; Bakkar, 1930; Mahmoud and Malik, 1978; Wells, 1982).

Irrespective of these facts, research in and control campaigns against *T. evansi* have not attracted the degree of coordination, discussion and international cooperation comparable to its tsetse-transmitted counterparts. However, the early recognition of the importance of its infection as a serious constraint to livestock development and food crop production prompted countries such as India as far back as 1940, to establish centres for the monitoring of the infections and treatment of infected livestock (Luckins, 1988). In addition, recent awareness of, and increased interest in, the significance of *T. evansi* in livestock development and food production has motivated the establishment of an international working group of *T. evansi* by the Office Internationale des Epizooties (OIE), which provides a regular opportunity for researchers and field workers to discuss recent developments in the epizootiology, treatment, immunology and economic significance of the parasite.

Despite such international collaborative efforts on *T. evansi* and notwithstanding the large body of information currently available on various aspects of the tsetse-transmitted trypanosomes, the scourge of trypanosomosis remains. Part of the reason for this is that unlike many bacterial and viral diseases, the prospect of controlling the infection by the development and administration of vaccines using trypanosomes or their products remains an elusive and remote dream. This is because of the antigenic diversity of the parasites and their ability to vary antigens during the course of infection (Vickerman, 1969; Gray and Luckins, 1976; Cross, 1978; 1979b; 1990). This phenomenon, known as antigenic variation, precludes the development of an immunoprophylactic agent, and largely accounts for the success of the organisms as parasites (Ritz, 1916; Gray, 1965a).

Antigenic variation, whereby trypanosomes sequentially express and shed a series of different surface glycoproteins (VSGs) enables them to evade the hosts protective immune responses. These surface antigens, upon contact with the hosts immune effector cells, induce a rapid and highly efficient immune response capable of destroying the trypanosomes. However, this response is also highly specific for trypanosomes possessing a particular VSG and invariably fails to affect any trypanosomes having different VSGs (Murray *et al.*, 1979) which then repopulate the infected hosts in cycles of parasitaemic waves. In addition, trypanosomes produce alterations in the functioning of the hosts immune system which can result in a state of generalised immunosuppression. Thus, not only are infected animals unable to reach a state of sterile immunity against the invading trypanosomes but

they might also eventually fail to mount specific protective immunity against other pathogens to which they become more susceptible. The implication and consequences of this phenomenon in trypanosome enzootic areas can be very serious since in the tropical and subtropical regions, many diseases occur in domestic animals and control depends on massive vaccination campaigns.

The first experimental evidence of trypanosome-induced immunosuppression came from the observation that *T. brucei*-infected mice showed impaired antibody response to sheep red blood cells [SRBC] (Goodwin, 1970, Goodwin *et al.*, 1972). This was corroborated by several other workers establishing immunosuppression as an important feature of African trypanosomosis (Holmes *et al.*, 1974; Murray *et al.*, 1974a; 1974b; Corsini *et al.*, 1977; Roelants *et al.*, 1979a, b). However, it was not clear how, and by what means, trypanosome infection caused immunosuppression. Thus, the mechanism of trypanosome-induced immunosuppression became a completely new chapter on the subject which in the past two decades has been intensively researched. Various studies have shown that polyclonal B cell activation, generation of suppressor T cells and macrophages and altered antigen handling and presentation (Esuruoso, 1976; Eardley and Jayawardena, 1977; Mansfield and Bagasra, 1978; Roelants and Pinder, 1984) are all mechanisms which could be involved in trypanosome mediated immunosuppression. However, the bulk of these studies have been done on laboratory rodents using either *T. brucei*, *T. congolense*, *T. gambiense* or *T. rhodesiense* and have concentrated on *in vitro* studies of responses of either spleen or lymph node cells from infected and uninfected rodents to various mitogens. So far, no comparable work in rodents has been done using *T. evansi* and information is lacking on this important aspect of the immunopathology of *T. evansi* infection.

As regards the tsetse-borne trypanosomoses, little work has been done on generalised immunosuppression in livestock. Hence, it is not clear whether the immunosuppression seen in experimental rodents and the mechanisms suggested for its induction occur also in infected livestock. There is even less information available on the subject in the case of *T. evansi* infected livestock. In any case, considerable differences exist between the pathogenic effects of infection in mice and cattle. It was thought therefore, that immunosuppression might not be a feature of livestock trypanosomosis, especially as the enlargement and destruction of lymphoid architecture in large animals is not as dramatic as in rodent trypanosomosis. However, limited studies have been carried out to determine the immune status in infected livestock and the results have not been unequivocal (reviewed by Vickerman and Barry, 1982; Urquhart and Holmes, 1987). Although, often conflicting, evidence points to suppressed immune responses to various bacterial and viral vaccine

antigens. For example, profound reductions in antibody responses to a polyvalent clostridial vaccine were seen in cattle experimentally infected with *T. congolense* (Holmes *et al.*, 1974), to louping-ill vaccine in cattle infected with *T. brucei*, *T. congolense* and *T. vivax* (Whitelaw *et al.*, 1979) and to *Brucella abortus* vaccine in cattle infected with *T. congolense* (Rurangirwa *et al.*, 1983). In contrast, with foot and mouth disease (FMD) vaccine, Scott *et al.* (1977) showed that although the antibody responses were reduced in cattle harbouring natural trypanosome infection, almost all antibody titres were above those considered necessary to give more than 95 percent protection against challenge with infectious virus within 10 days of the second vaccination. This result was corroborated by results of a similar work in which cattle, experimentally infected with *T. congolense*, and vaccinated with FMD vaccine were subsequently challenged with the live virus (Sharpe *et al.*, 1982). This suggests that while there is distinct evidence for a quantitative reduction of anti-FMDV antibody response in the parasite-infected cattle, it is unlikely to interfere with the effectiveness of the vaccination programmes. However, the possibility that such immunosuppression may be prejudicial to an adequate duration of immunity is likely since a combination of accelerated immunoglobulin catabolism (Nielsen *et al.*, 1978a) and lower antibody titres is unlikely to be conducive to a sustained, efficient immune response. Moreover, in animals existing on a marginal plane of nutrition, subjected to repeated trypanosome challenge and exposed to a wide variety of other pathogens, even a modest reduction of the antibody levels could have serious effects on efficient immune responses to vaccines as well as on resistance to the trypanosomes and other pathogens (Urquhart and Holmes, 1987). It is pertinent to emphasize that this fact is often overlooked in considerations of the impact of trypanosomosis on livestock performance in Africa and other regions of the world where the disease is a problem. Significantly, it is also a fact that livestock in most of these areas is more likely to be kept on a marginal nutritional plane, trekked for long distances in search of water and pasture and exposed to a variety of other stress factors. Such conditions amplify the effects of trypanosome infections which may therefore render futile any attempts at controlling other diseases by vaccination.

Marked lymphoid organ hyperplasia, altered cellular demographics and hypergammaglobulinaemia are consistent features of trypanosomosis in large animals which may have significant implications in the immunopathology of the disease. Despite this, however, the mechanisms of the trypanosome-induced immune dysfunction in domestic livestock are more obscure and ill-defined than in rodents. It may be possible that some of the mechanisms suggested for laboratory rodents also operate in large animals. The few studies in which the response to

mitogen of leucocytes from infected cattle were examined failed to demonstrate any evidence of dysfunction (Sollod and Frank, 1979; Masake *et al.*, 1981; Masake and Morrison, 1981). In contrast, Emery, *et al*. (1980c) and Morrison *et al.* (1985) were able to demonstrate *in vitro* proliferative responses of peripheral blood leucocytes (PBLs) to ultrasonicated trypanosomal antigen in cattle which had been cured of trypanosome infection by chemotherapy. However PBLs taken from infected animals showed no response. Thus they argued that the effector cells of infected cattle were sensitised to trypanosome antigen(s) but their proliferative response was suppressed during infection. Although not presented, they claimed in conclusion that preliminary results from their laboratory indicated that the suppressed responses were those of T cells (Morrison *et al.*, 1985). Similarly, PBLs from *T. evansi*-infected ponies failed to respond to stimulation *in vitro* with either living *T. evansi*, soluble *T. evansi* antigens or isolated VSG. Following treatment, cells from these animals responded only to large amounts of the soluble antigens while living *T. evansi* and purified VSG had no effect (Ahmed *et al.*, 1988; 1990).

Detailed studies of the cytological changes in infected livestock and their immunological implications was not possible until recently due to the lack of cell typing reagents for large animals. This obstacle has been overcome by hybridoma technology and the development of monoclonal antibodies (mAbs) to cell surface antigens which has revolutionised the approach and techniques of studying the immune systems and responses of mammals. These mAbs have permitted the identification of the surface molecules (antigens) of immune cells and their characterisation into functionally distinct subsets recently codified as cluster of differentiation (CD) antigens. These differentiation antigens in normal immunocompetent vertebrates are expressed in various proportions and ratios which allows them to cooperate in association with accessory cells in mounting a specific sterile immune response against foreign antigens. The recent development of mAbs with specificities for bovine leucocyte surface antigens (Baldwin *et al.*, 1986; Ellis *et al.*, 1986; Lalor *et al.*, 1986; Davis *et al.*, 1988) and ovine leucocyte surface antigens (Gogolin-Ewens *et al.*, 1985; Mackay *et al.*, 1985; 1986; 1987; Maddox *et al.*, 1985a, b; Puri *et al.*, 1985; Hopkins *et al.*, 1986) has allowed the identification and characterisation of the human and murine analogues of various lymphocyte CD antigens in these species. Thus using these mAbs, attempts have been made to study the dynamics and roles of different lymphocyte subsets in immune responses in various diseases in which immune dysfunction is a feature. It has been revealed through such studies that there is significant alteration in the expression kinetics of the lymphocyte phenotypes in disease and that particular alterations may modulate the immunological resistance of the host to the disease. A classic example of the

immunological implication of altered expression of lymphocyte CD antigens in infection is the depletion of the $CD4^+$ T cells by the human immunodeficiency virus (HIV) with an accompanied increase in the expression of $CD8^+$ T cells which results in subsequent destruction of the patient's immune defences and the development of the acquired immunodeficiency syndrome [AIDS] (Kornfeld *et al.*, 1982; Fahey *et al.*, 1984; Cavaille-Coll *et al.*, 1984; Lewis *et al.*, 1985; Melbye *et al.*, 1986; Cooper *et al.*, 1988; Edelman and Zolla-Pazner, 1989; Aiuti *et al.*, 1989). Similarly, there is marked alteration in the expression and ratios of $CD4^+$ and $CD8^+$ T cell subsets in patients suffering from lepromatous leprosy (Bach *et al.*, 1981).

As in African human trypanosomosis, Chagas' disease caused by *T. cruzi* is also accompanied by severe immunosuppression (Teixeira *et al.*, 1978; Voltarelli, Donadi and Falcao, 1987) which undermines resistance to the establishment and dissemination of the organism in man. In trying to elucidate the mechanisms involved in *T. cruzi*-induced immunosuppression, Beltz *et al.* (1988) and Stein *et al.* (1990) provided evidence that altered expression of certain lymphocyte surface molecules was responsible for the immune dysfunction seen. Using *in vitro* culture systems they demonstrated that *T. cruzi* induced the suppression of interleukin 2 receptor (IL-2R) expression by phytohaemagglutinin (PHA) or monoclonal anti-CD3 stimulated human peripheral blood mononuclear cells (PBMC). This *T. cruzi*-mediated suppression of IL-2R expression was also accompanied by, and correlated with, a marked reduction in the expression of surface CD3, CD4 and CD8 as well as a significant decrease in the percentage of $CD3^+$, $CD4^+$ and $CD8^+$ cells. CD3 is a critical component of the TcR-CD3 complex which is involved in transduction of signals generated by specific antigen binding. CD4 and CD8 apart from contributing to lymphocyte activation through interaction with the TcR-CD3 complex (Allison and Lanier, 1987; Schrezenmeier and Fleischer, 1988; Parnes, 1989) are also involved critically with MHC interaction and signal transduction. IL-2R expression by $CD4^+$ and $CD8^+$ cells is required for lymphocyte activation and for activated lymphocytes to be able to complete their cell division and function properly. Thus, inhibition of expression of IL-2R, CD3, CD4 and CD8 molecules by T cells underlies the immunosuppression seen in Chagas' disease, since the immunological functions of B and natural killer cells would be adversely affected, thereby extending the repercussions of the initial T cell defects (Kierszenbaum and Sztein, 1990).

Limited attempts have been made recently to determine the dynamics of lymphocyte phenotypes in trypanosome-infected livestock and assess their possible immunological roles. Comparative studies of the circulating PBL subpopulations in both trypanotolerant N'dama and trypanosusceptible Boran breeds of cattle exposed

to tsetse-transmitted infection with *T. congolense*, showed that N'dama had significantly higher numbers of B cells than Boran although both breeds showed parallel significant decreases in the number of $CD4^+$ and $CD8^+$ T cells from the pre-infection levels to the first peak of parasitaemia (Ellis *et al.*, 1987). In contrast, Williams *et al.* (1991) found no differences in the increase in the number of circulating B cells in either N'dama and Boran cattle rechallenged with homologous *T. congolense*. However, significant differences were observed between the two breeds in the number of $CD4^+$ T cells which decreased in the Boran group while remaining within pre-infection levels in the N'dama. In addition, the CD5 antigen was detected on 50-90 percent of circulating B cells in all infected animals compared with 5-10 percent of B cells in uninfected animals. Mwangi (1991) has also demonstrated marked decreases in all T cell subsets as well as increases in B cells in the PBL of sheep experimentally infected with cultured metacyclic *T. congolense*. Changes in the kinetics of various lymphocyte subsets were also observed in the skin, lymph nodes and peripheral afferent and efferent lymph draining from the site of trypanosome challenge (Mwangi, Hopkins and Luckins, 1990; Mwangi, 1991). No comparable work has so far been done employing *T. evansi* in any of its varied livestock hosts.

The above studies have provided useful insights into the cellular alterations which might underlie trypanosome-induced immunosuppression in domestic livestock. However, the changes in cellular phenotypes were not studied in association with humoral responses either to the trypanosomes themselves or to a heterologous antigen challenge during the infection. Such studies could provide better understanding of whether, and how, the alterations in the immune effector molecules affect the generation of sterile immunity.

This thesis is concerned with trypanosome-induced immunosuppression and its mechanisms in domestic livestock. *T. evansi* is naturally infective to the sheep in which it produces a chronic infection. The sheep-*T. evansi* model therefore offers an opportunity for extended studies of disease without recourse to chemotherapy. The experiments make use of *P. haemolytica* serotype A1 vaccine suspended in aluminium hydroxide gel (alhydrogel) as a model antigen because of its high immunogenicity (Gilmour *et al.*, 1979). Four different criteria of immunological responsiveness were examined:

1. Cell kinetics and phenotypes using a panel of mAbs with specificities for ovine lymphocyte antigens firstly, in sheep infected with *T. evansi* and secondly in infected and uninfected sheep following primary and secondary vaccine challenge.
2. Changes at the site of vaccine antigen inoculation, examining

inflammatory and hypersensitivity reactions.

3. Measurement of cellular reactivity to the model antigens as assessed by *in vitro* proliferative assays and

4. Measurement of specific anti-trypanosomal and anti-*Pasteurella* antibodies in serum.

Hence, the aim of these studies is to investigate whether *T. evansi* causes immunosuppression in one of its natural hosts and if so to explore the possible mechanism(s) by which it mediates that suppression.

CHAPTER TWO

Literature Review

SECTION A

The Pathogenic African Trypanosomes

2A 1 Historical Background, Classification and Speciation of Trypanosomes

2A 1.1 Historical Background

Trypanosomes are eukaryotic digenetic protozoan parasites believed to have been recognised by scientists more than 300 years ago. Valentin (1841) observed what is believed to be the first trypanosomes in the blood of trout (*Salmo fario*), which he described as motile elongated organisms, and likened them to amoebae. However, the true nature of his organism was disputed by other workers including Gluge (1842) and Mayer (1843) who observed flagellates in the blood of various amphibians. At the same time Gruby (1843) observed and studied these amphibian haemoparasites and created the generic name *Trypanosoma* (*Tripanosoma*) for them.

The first mammalian trypanosomes were discovered by Lewis (1878) in the blood of black and brown rats in India. These parasites, which were later named *Trypanosoma lewisi* proved to be cosmopolitan, non-pathogenic parasites of their mammalian hosts. The discovery of the first pathogenic mammalian trypanosomes was also made in India by Griffith Evans in 1880 while investigating a disease of horses known by the local Indians as 'Surra' (meaning low and rotten in spirit). He observed the parasites in the blood of horses suffering from surra and implicated them as the aetiological agent of the disease. Evans' pioneering work (Evans, 1880; 1881-1882) was ridiculed, largely because not only was the 'microbial' nature of diseases still very controversial and then in its infancy, but also because the parasite he described resembled the inocuous commensal previously described by Lewis in rats. Notwithstanding, Evans proved beyond doubt that the trypanosomes he saw were pathogenic to domestic animals. Although his work stimulated little interest at the time, it undoubtedly was the basis of all subsequent investigations which incriminated other species of trypanosomes as the causative agents of human and animal trypanosomoses. Evans described unequivocally the clinical disease and gross pathology in a number of different animal hosts. He also demonstrated experimentally that the disease was transmitted by haematophagous flies (Evans, 1880; 1881-1882). This finding was corroborated by Rogers (1901) whose work also proved that the transmission was mechanical in nature. Bruce (1895) working in Africa, investigated a cattle disease in Zululand called 'Nagana' and reported "the constant occurrence in the blood, of an infusorial parasite either identical with or closely resembling the *T. evansi* found in Surra, a disease of India and Burma". He also claimed incorrectly, that the trypanosomes (later named *Trypanosoma brucei* (*brucii*) by Plimmer and Bradford (1899) were mechanically transmitted by tsetse flies. It was Kleine (1909) who showed that the transmission of pathogenic African

trypanosomes is not mechanical but biological since they undergo cyclical development in the tsetse fly vector prior to transmission.

Bruce's discovery was followed by those of Dutton (1902) and of Stephens and Fantham (1910) who discovered trypanosomes in human patients suffering from sleeping sickness in West Africa and Rhodesia and which they named *T. gambiense* and *T. rhodesiense* respectively. Earlier in that decade, Doflein (1901) proposed the name *T. equiperdum* for the causative agent of Dourine in horses whose nature and disease had been earlier described by Rouget (1896) in Algeria. In the Congo, an unusually small parasite lacking a free flagellum was discovered in the blood of sheep and a donkey and named *T. congolense* by Broden (1904). *T. vivax* and *T. suis* were discovered by Ziemann (1905) and Ochmann (1905) respectively, while a pathogenic pig trypanosome was first described by Montgomery and Kinghorn (1909) and was named *T. simiae* by Bruce *et al.* (1912). Also in 1909, Carlos Chagas discovered an epimastigote form of a flagellate in the gut of a Reduviid bug (*Panstrongylus megistus*) and showed that monkeys fed on by these bugs developed trypanosome (*T. cruzi*) infection. Chagas suspected that the trypanosomes might be responsible for a disease (Chagas' disease) of unknown aetiology which afflicted the local people. He searched for the parasite in human subjects, finally finding it in a child living in a bug-infested house. *T. cruzi* thus became one of the first examples of a parasite discovered in its insect vector before its vertebrate host became known.

2A 1.2 Classification

The taxomony and systematic classification of trypanosomes since Gruby (1843) created the genus *Trypanosoma* has presented zoologists, systematists and geneticists with its share of controversy and criticism. Thus, while over a dozen generic names have been proposed for trypanosomes the generic name *Trypanosoma* has retained its taxonomic status as the valid type-genus with all other proposed generic names falling into oblivion. According to the classification proposed by the Committee on Systematics and Evolution of the Society of Protozoologists, published by Levine *et al.* (1980) and cited by Soulsby (1982), the systematic position of pathogenic trypanosomes is as follows:

SUB KINGDOM:	PROTOZOA Goldfuss, 1818; *emend.* Sielbold, 1845
PHYLUM:	SARCOMASTIGOPHORA Honigberg & Balamuth, 1963
SUBPHYLUM:	MASTIGOPHORA Diesing, 1866
CLASS:	ZOOMASTIGOPHOREA Calkins, 1909
ORDER:	KINETOPLASTIDA Honigberg, 1963
SUBORDER:	TRYPANOSOMATINA Kent, 1880

FAMILY: TRYPANOSOMATIDAE Doflein, 1901; *emend.* Grobben, 1905

GENUS: TRYPANOSOMA Gruby, 1843

2A 1.3 Speciation

With the general acceptance of *Trypanosoma* as the valid generic name, the speciation and accordance of formal recognition to the heterogeneity of trypanosome organisms remained for sometime controversial since the concept of species is classically restricted to organisms with sexual reproduction. Although trypanosomes are agamous reproducers, Hoare (1972) has argued that the difficulty of assigning species status to them can be overcome by redefining 'species' in general terms as 'assemblages of organisms descended from a common ancestor and segregated from other populations by natural selection, determined by the environment and struggle for existence'. Therefore, since trypanosomes can be grouped into assemblages or biotypes of similar individuals, which presumably possess a closely related genetic pattern and occupy a common habitat (= hosts) and/or area of distribution (= host range), they satisfy the fundamental requirements for the recognition of a population as species. Thus, well-defined clones of trypanosomes are entitled to be classified in the conventional manner (Poljansky, 1957; Dogiel *et al.*, 1965).

The first real attempt to differentiate between the diverse mammalian trypanosomes was made by Laveran (1911) who grouped the organisms according to their pathogenicity. However, the first sound biological criteria for the speciation of pathogenic trypanosomes were independently proposed by Duke (1913) and Roubaud (1913) and entailed the separation of trypanosomes into three groups according to their mode of development. Bruce (1914), as well as Knuth and du Toit (1921) improved and extended these criteria thus enabling the non-tsetse transmitted *T. evansi* and *T. equiperdum* to be grouped with the tsetse transmitted *T. brucei*. The work of these authors was later collated and rationalised by Wenyon (1926) who grouped the different species according to the site of termination of their development in the vector. Hoare (1957; 1964; 1966), drew upon these earlier studies and developed a classification of trypanosomes on phylogenetic lines based on both morphological and biological criteria. This mode of classification not only gave expression to the affinities of the trypanosomes but also brought together related species that exhibited similarities in their physiology, antigenic constitution and host-parasite relationship. In the case of pathogenic trypanosomes, these characteristics have direct bearing on their response to chemotherapy, on the immunological reactions of the host and on the clinical manifestation of the diseases caused by them (Hoare, 1972).

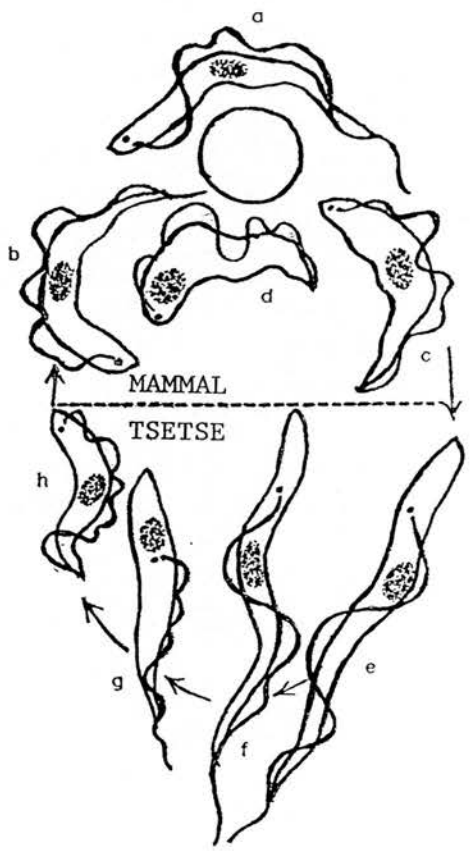


FIGURE 2.1 Developmental forms of salivarian trypanosomes (*Trypanosoma (Trypanozoon) brucei*). a-d. Bloodstream trypomastigote forms: a. Slender form; b. Intermediate form; c. Stumpy form; d. Stumpy posterionuclear form. e-h. Developmental stages in Tsetse fly: e. Trypomastigote in fly stomach; f. Proventricular trypomastigote (Promastigote) in cardia; g. Epimastigote and h. Metacyclic forms in the salivary gland. (Redrawn from Hoare, 1949).

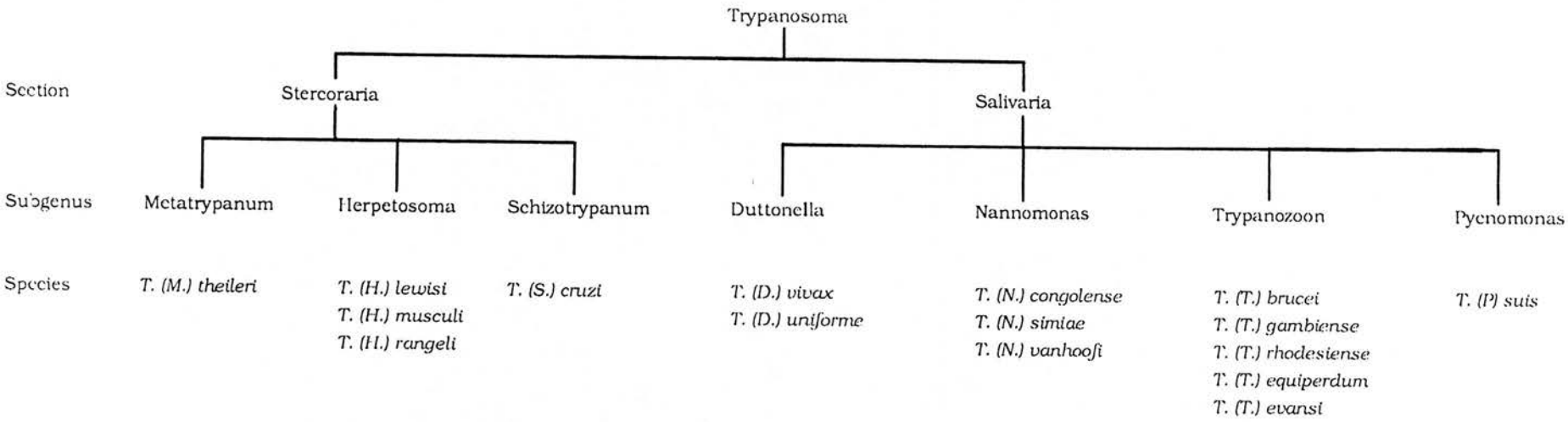
Mammalian trypanosomes fall naturally into two major groups known as Section A and B. These are distinguished by their mode of development primarily in the vector and secondarily in their mammalian host. Hoare (1964) applied the descriptive terms of Stercoraria and Salivaria to the two sections respectively. Section A (Stercoraria) contains species in which the entire invertebrate cycle of development is confined to the gut of the vector with the final production of infective metatrypanosomes in the faecal medium of the hind-gut. Transmission from the faeces of the insect to the body of the primary host is contaminative and trypanosomes belonging to this group are said to undergo 'posterior station' development. Morphologically, a free flagellum is always present, kinetoplasts are large, posterior end is generally pointed and multiplication in the mammalian host is discontinuous and usually occurs in the amastigote or epimastigote stages. Section B (Salivaria) comprises species whose cyclical development in the vector is completed in the 'anterior station'; that is to say, only the initial stages of development take place in the mid-gut of the vector. The final stages, which terminate in the production of the infective trypanosomes (Figure 2.1), occur either in the proboscis or the salivary glands of the vector. The entire invertebrate cycle of development for *T. vivax* and *T. uniforme* is restricted to the proboscis. Transmission to the mammalian host is by the inoculative means through the bite of an infected fly. Morphologically (Table 2.1) a free flagellum may or may not be present. The kinetoplast is relatively small, terminal or sub-terminal, the posterior end is often blunt and multiplication in the vertebrate host is continuous in the trypomastigote stage (Figure) 2.1. Most pathogenic African trypanosomes belong to this section. Details of the speciation of members of the genus *Trypanosoma* is given in Table 2.2 and is based on Molyneux and Ashford (1983). As can be seen, even this system is not wholly satisfactory since non-pathogenic trypanosomes such as *T. lewisi*, are grouped with pathogenic *T. cruzi* while *T. evansi* and *T. equiperdum* transmitted mechanically and by coitus respectively are grouped with cyclically transmitted *T. congolense*, *T. vivax* and *T. simiae*.

However, analysis of their biochemical relationships based on electrophoretic patterns of various isoenzymes (Godfrey and Kilgour, 1976; Gibson, Marshall and Godfrey, 1980) and restriction endonuclease analysis of mitochondrial (kinetoplast) DNA have been used to characterise species of the *T. brucei* group (Borst *et al.*, 1980; 1982). In addition, the development and use of species-specific DNA probes (Massamba and Williams, 1984; Gibson, Dukes and Gashumba, 1988) and species-specific monoclonal antibodies (Nantulya *et al.*, 1987; Frame, 1989) is likely to provide more precise techniques for speciation and subspeciation within the different subgenera of trypanosomes.

Table 2.1 **Some morphological differences in African trypanosomes**

Morphological Characteristics					
Subgenera	Species	Size	Kinetoplast	Free flagellum	Undulating membrane
Duttonella	*T. vivax* *T. uniforme*	18-31 μ	Kinetoplast Large and terminally placed	Present in all stages	Inconspicuous
Nannomonas	*T. congolense* *T. simiae*	8-24 μ	Kinetoplast medium, marginal and subterminal	Absent in all stages	Inconspicuous
Trypanozoon	*T. brucei brucei* *T.b. rhodesiense* *T.b. gambiense*	17-27 μ	Kinetoplast small, subterminal	Present in all stages except infective forms	Conspicuous
Pycnomonas	*T. suis*	9-19 μ	Kinetoplast small, subterminal and marginal	Present in all stages	Inconspicuous

Table 2.2 **Classification of the genus *Trypanosoma* (after Molyneux and Ashford, 1983)**



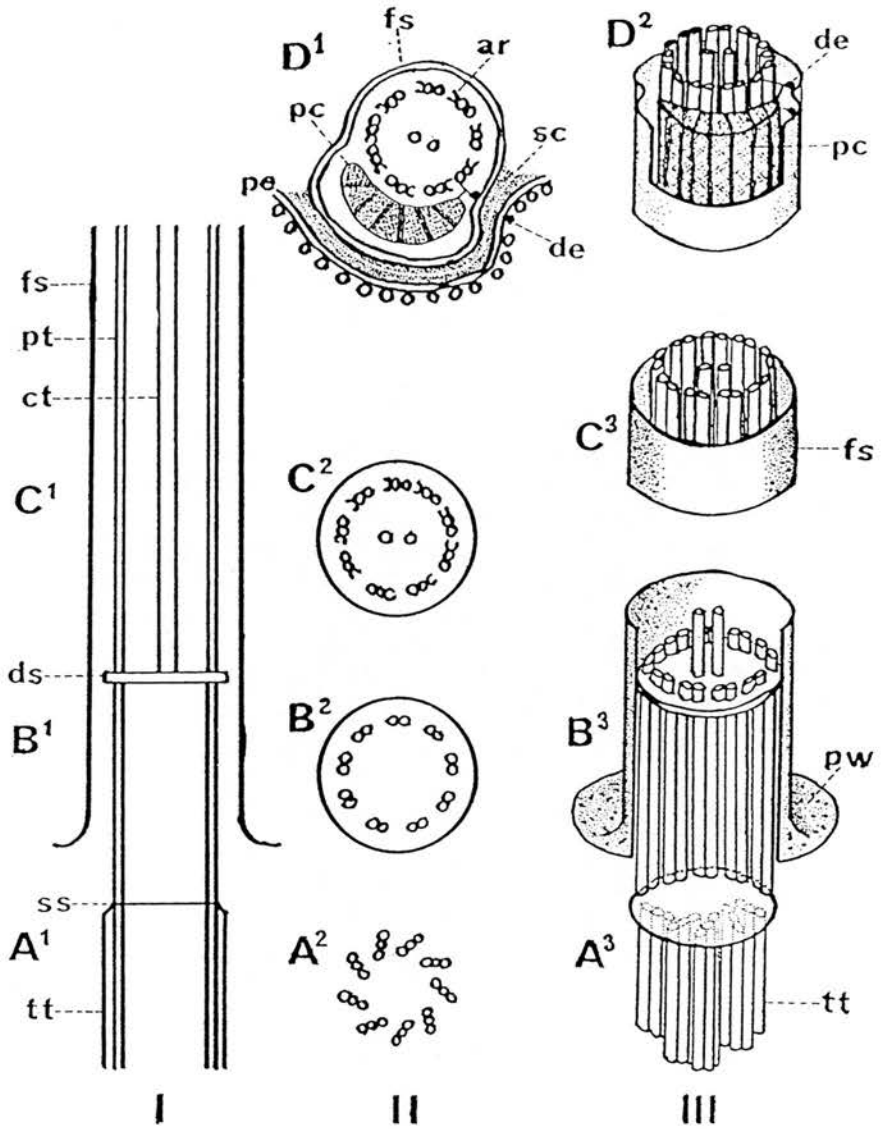


FIGURE 2.2a. **Ultrastructural features of the trypanosome: The Flagellum**

I. Longitudinal plane
II. Transverse sections, showing arrangement of microtubules
III. Three-dimensional view of longitudinal section in various zones
A^1-A^3. Basal body: intracytoplasmic zone
B^1-B^3. Basal body: extracytoplasmic (transitional) zone
C^1-C^3, D^1, D^2. Axoneme or flagellum seen at proximal (**C^3**) and distal (**D^2**) **parts**
D^1. Attachment of flagellum to body, with desmosomes (de) and surface coat (**sc**)
ct. central microtubules; **fs**. flagellar sheath; **pt**. peripheral doublet microtubules; **tt**. triplet microtubules. **(adapted from Hoare, 1972).**

2A 2 The Parasite and the Disease

2A 2.1 Morphology

2A 2.1.1 General Morphology of Trypanosomes

Much of the knowledge of the morphology of mammalian trypanosomes has been based on examination of dry-fixed blood film preparations stained by the Romanovsky method. This technique has revealed that typical bloodstream forms of mammalian trypanosomes have an elongated, spindle-shaped and flattened body which is usually curved and varies in length from 8-39 μm (Table 2.1; Mulligan, 1970; Hoare, 1972). These features vary according to species and can be modulated by the orientation of the parasite at the time of fixation during the slide preparation and staining. Organelles such as the flagellum, the kinetoplast and the nucleus can also be demonstrated by this method. However, much greater detail of the cytomorphology of the mammalian trypanosomes has been obtained through more recent studies using phase contrast and electron microscopy. These studies have shown that the trypomastigote is encased in a strong and resistant three-layer membranous envelope known as the pellicle (Figure 2.2a: D^1,pe). This membrane is enveloped by a surface coat (Figure 2.2a: D^1,sc) of amorphous material which is the location of the variable antigens present in the metacyclic and bloodstream forms (Rudzinska and Vickerman, 1968; Vickerman, 1970).

The flagellum (Figure 2.2a) represents the organ of locomotion and is a whip-like filamentous structure shown by EM to be composed of two parallel rod-like structures, the axial filament complex or axoneme and the accessory filament complex or paraxial cord, all of which are encased in a flagellar sheath (Figure 2.2a). The axoneme consists of nine peripheral doublet and two single central microtubules (Figure 2.2a, C^1-C^3,B^3). It originates from the basal body (also known as the blepharoplast or kinetosome) whose form and overall plan are akin to the flagellum, except for the presence of nine peripheral triplet and the absence of the two central microtubules (Figure 2.2a, A^1-A^3;B^1,B^2). The paraxial cord (Figure 2.2a, D^1pc;D^2pc) occurs only in the region where the flagellum emerges to form the undulating membrane and it is thought to strengthen the propulsive action of the flagellum (Hoare, 1972).

The kinetoplast, when seen under light microscopy (LM), may appear disc-shaped, round or rod-shaped and its shape and size as well as the position in the body is characteristic in certain species. It is always situated close to the basal body. EM has revealed that the kinetoplast is a DNA-containing disc of longitudinally orientated fibres enveloped by a two membrane capsule. It is continuous with, and forms an integral part of, a canal system. It has a typical mitochondrial structure with the inner membrane of the double membranous wall

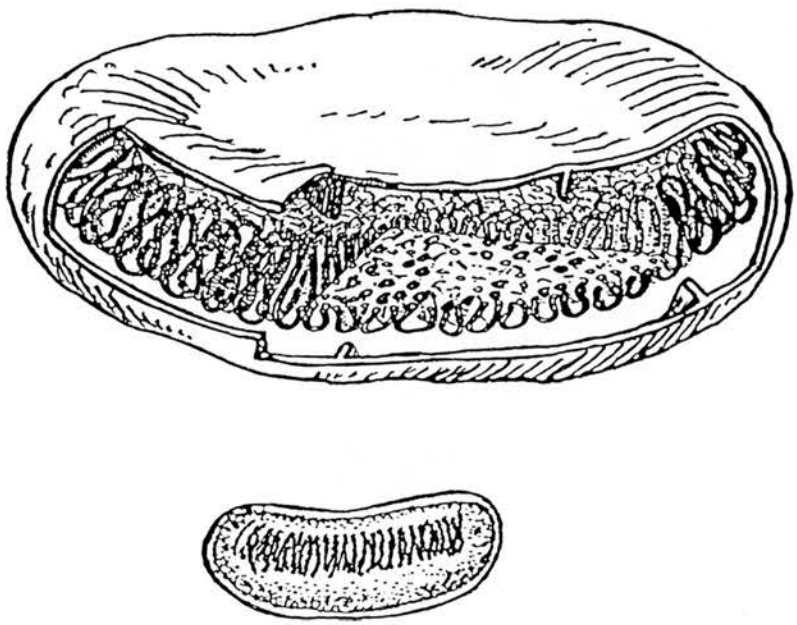


FIGURE 2.2b. **Ultrastructure of the trypanosome: The Kinetoplast** (*T. evansi*)

i. Schematic ultrastructure of the kinetoplast mitochondrion in three dimensional view showing the DNA-fibrils drawn into cristae.

ii. *T. evansi* kinetoplast as revealed by electron microscopy **(adapted from Hoare, 1972).**

drawn into cristae (Figure 2.2b; Clark and Wallace, 1960; Vickerman, 1965; Vickerman and Tetley, 1979). The single mitochondrion which extends the entire length of the organism (Figure 2.2c/2.2d) has the primary function of oxidative phosphorylation and provision of energy for the parasite. The complexity of the structure of this organelle and the degree of the function it performs depends on whether the parasite is in the bloodstream or in the fly. Bloodstream forms derive much energy from massive glucose and oxygen supply within the host's blood and thus have simple mitochondrion of a single canal tube and very few cristae (Figure 2.2c). Fly midgut forms, which find themselves in stagnant blood and diminishing glucose and oxygen supply develop an expanded and functionally more active mitochondrial network with more branches and abundant cristae (Figure 2.2d; Vickerman, 1965; 1970; Vickerman and Tetley, 1977).

The nucleus as seen under LM is a vesicular body, spherical or ellipsoidal in shape and is bounded by a nuclear membrane. EM has revealed that the nuclear membrane is double layered and punctured by pores with the outer layer being continuous with the endoplasmic reticulum (Figure 2.2c). There is a dense mass at the centre of the nucleus which is the nucleolus (endosome or karyosome). This consists of closely packed electron-dense ribosome-like granules. Ribosomes are also scattered throughout the cytoplasmic matrix (Vickerman, 1970).

2A 2.1.2 Morphology of *T. evansi*

In the blood of its mammalian hosts *T. evansi* is represented almost exclusively by long, thin trypomastigotes comprising the slender and intermediate forms indistinguishable from those of the bloodstream forms of *T. brucei* and the allied human parasites. Although typically monomorphic, there is evidence that some strains occasionally exhibit the same type and degree of pleomorphism as *T. brucei* (Bruce, 1911; Frior *et al.*, 1916; Velu, 1918; Lavier, 1933; Hoare, 1956) including the presence of typical posteronuclear stumpy forms (Figure 2.2e). However, since stumpy forms are usually absent or occur only sporadically and in insignificant numbers (Hoare, 1972), in typical strains, *T. evansi* is almost exclusively monomorphic. There is a long free flagellum and a more or less drawn-out narrow posterior extremity. This may be rounded or truncated, with the kinetoplast situated at some distance (subterminal position) from the tip (Figure 2.2e). The intermediate forms have a shorter free flagellum and a short, frequently pointed posterior extremity, with the kinetoplast lying near to this end (marginal position, Figure 2.2e). The kinetoplast, as in other trypanosomes of the sub-genus *Trypanozoon*, is small and usually rod-shaped. Sometimes mutant individuals occur in which the organelle appears to be lacking. These sometimes give rise to

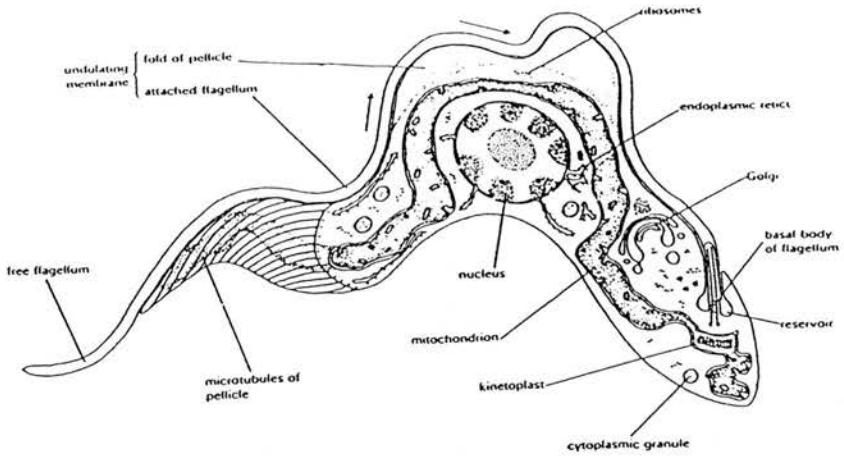


FIGURE 2.2c Diagram of trypomastigote intermediate bloodstream form of *Trypanosoma rhodesiense* as seen at the level of the electron microscope. The arrows along the flagellum indicate the direction of travel of flagellar waves. Pellicular microtubules are shown only at the anterior end of the flagellate. Note the single mitochondrial canal **(after Vickerman, 1970).**

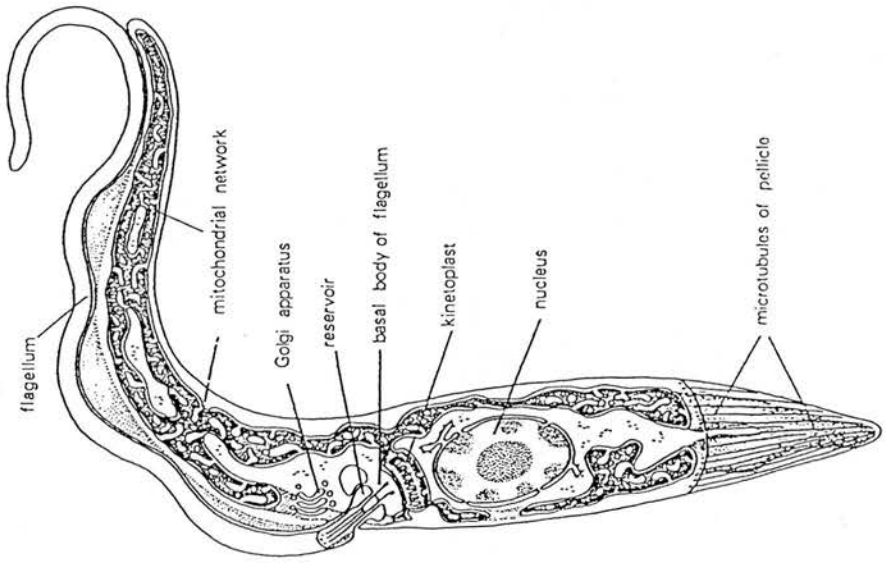


FIGURE 2.2d Diagram of epimastigote stage of *Trypanosoma rhodesiense* as seen at the level of the electron microscope. Pellicular microtubules are shown only at the posterior end of the body. Note that the mitochondrion is now a well developed network of canals **(after Vickerman, 1970).**

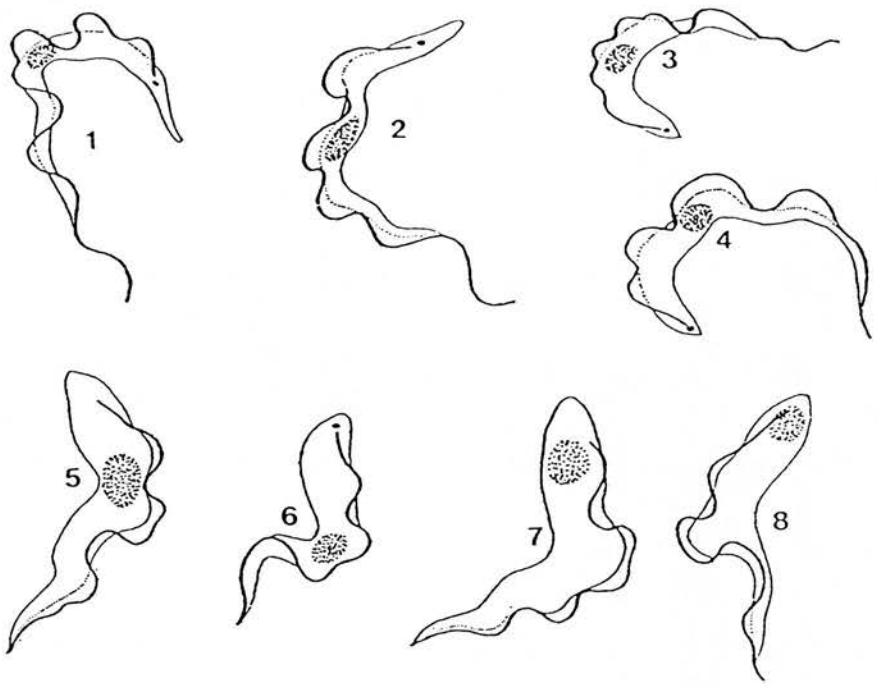


FIGURE 2.2e Morphological forms of *T. evansi*

1-2	Slender forms
3-4	Intermediate forms
5-6	Stumpy forms
7-8	Stumpy, posterionuclear, dyskinetoplastic forms

(adapted from Hoare, 1972).

permanently dyskinetoplastic strains (Figure 2.2e; Hoare and Bennet, 1937). There is considerable variation in the dimensions of various strains and populations of typical *T. evansi*, but its overall length distribution is 15-34 µm : mean = 24 µm (Hoare, 1956).

2A 2.2 Life Cycle and Transmission of *T. evansi*

Unlike tsetse-borne trypanosomes, whose life cycle involves a defined cycle of development in the insect vector prior to natural transmission, *T. evansi* does not undergo any cycle of development in an intermediate host before transmission. Multiplication of the parasite is restricted to the vertebrate host in which they proliferate by longitudinal binary fission in the trypomastigote stage.

T. evansi is therefore normally transmitted from infected to susceptible animals by mechanical inoculators represented by blood sucking Diptera other than *Glossina*, (especially Tabanid flies). The most convincing experimental confirmation of the role of mechanical inoculators as vectors of *T. evansi* was provided by Nieschulz (1930), who carried out comprehensive investigation on the subject in Indonesia, where equine surra was prevalent. He used thousands of insects, including species of *Tabanus, Chrysops, Haematopota, Stomoxys, Lyperosia, Musca* and some mosquitoes in these experiments. By feeding the insects on infected horses or buffaloes, then interrupting their meal at various time intervals and again feeding them on healthy horses, dogs and laboratory rodents, he demonstrated that the most efficient vectors were the horse-flies (*Tabanus* spp). The effectiveness of *Chrysops* and *Haematopota* was negligible while *Stomoxys* and *Lyperosia* failed to transmit the infection. However, in Africa, both the horse-flies and stable-flies (*Stomoxys*) have been incriminated in transmission of *T. evansi* (Barotte, 1925; Curasson, 1943). Also, since surra occurs in camels very close to the 'tsetse belt' in West Africa, Godfrey and Killick-Kendrick (1962) suspected that *T. evansi* might be capable of undergoing cyclical development in *Glossina*. However, it was demonstrated experimentally by Hoare (1948), who fed *G. morsitans* on animals infected with *T. evansi*, that they are incapable of developing in *Glossina* since the trypanosomes are killed in the gut of the flies during the first hours following ingestion.

As a rule, the shorter the interval between the two feeds by a vector, the greater the chances of successful mechanical transmission since this depends on the length of time the trypanosomes survive in the mouth-parts of the vector (Hoare, 1972). The longer the time within the vector, the less infectious the trypanosomes become. Even the most efficient vector, the horsefly, fails to successfully transmit *T. evansi* when the interval from the time of the infective feed on an infected host to the

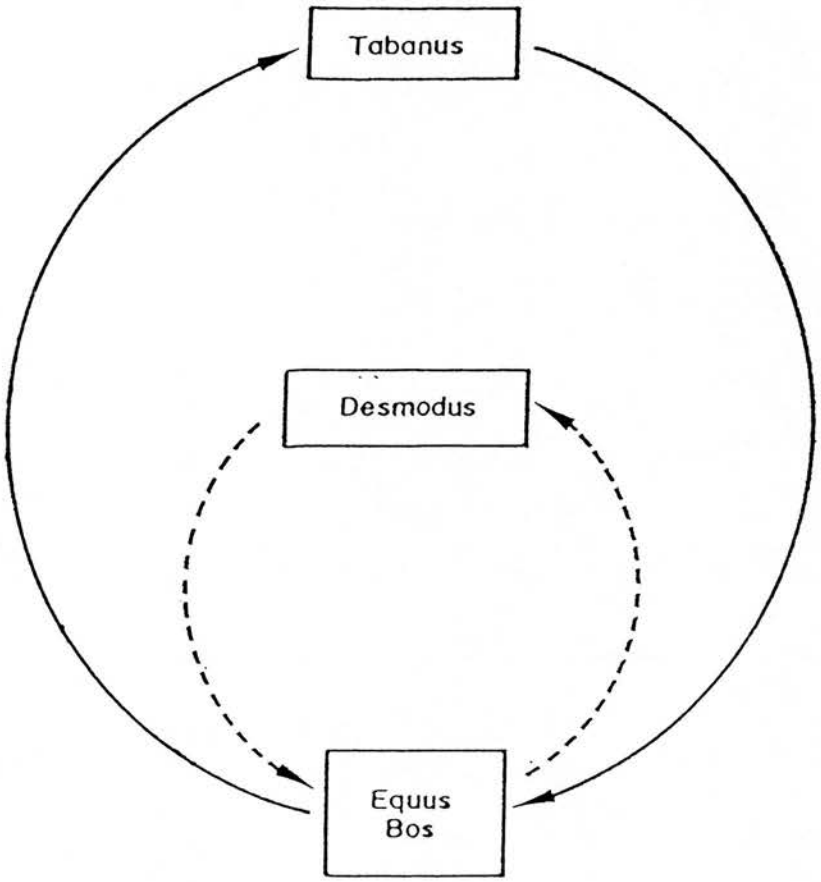


FIGURE 2.3 Diagram of life cycle of *Trypanosoma evansi* in Latin America. Circulation of the parasite is shown by arrows: continuous lines indicate main cycle (metaxeny) involving insect-vector; interrupted lines indicate parallel cycle (paraxeny) involving Vampire bat. **(From Hoare, 1965).**

time of the second feed on the susceptible host exceeds 8 hours.

In Central and South America, *T. evansi* is usually transmitted mechanically by Tabanid flies (Boehringer and Prosen, 1961). However, the maintenance and opportunity for transmission of the parasite is considerably enhanced by other vector species. In addition to biting flies, the sanguivorous vampire bat, *Desmodus rotundus* has been demonstrated to transmit this disease. These bats are ideal vectors, for even when feeding on hosts such as cattle with very scanty parasitaemia, their infection is ensured by the large amount of blood consumed, while the proliferation of the trypanosomes in their own blood increases the chances of successful transmission to new hosts. Thus Hoare (1965) pointed out that the host-parasite relations between the vampire bats and *T. evansi* present an unusual combination of vector and host in the same animal. The implication of this dual role is that the success of transmission of *T. evansi* by bats does not depend on the temporary survival of the parasites during the intervals between interrupted meals, rather, the establishment and persistence of the infection in the bats make them capable of transmitting the parasites for long periods of time to a wide range of hosts. Yet the bats differ from specific biological (cyclical) vectors in that the parasites do not undergo in them a development characteristic of a true vector but multiply in their blood in much the same manner as they do in the natural vertebrate host. Thus Hoare (1965) proposed two types of life cycle and transmission patterns for *T. evansi* in the region, known as metaxenic and paraxenic live cycles (Figure 2.3). Finally, the tranmission of *T. evansi* to carnivores, especially dogs, is effected by the ingestion of raw meat from infected animal carcasses (Mulligan, 1970).

2A 2.3 Host Range, Pathogenesis and Pathology of *T. evansi*

T. evansi has a wide host range in which it causes disease. Among domesticated animals it affects horses, donkeys, mules, camels, the Indian elephant, cattle, buffaloes, sheep, goats, dogs, cats and pigs (Stephen, 1986). In addition, it affects a wide range of wild animals including various felines, canines and ruminants. These wild animals are as much victims of the disease as domesticated livestock and they probably acquire the infection secondarily from domesticated animals. Many are highly susceptible to *T. evansi* which has severe pathological effects on them. They are therefore not normally regarded as reservoir hosts through which domestic animals are infected (Hoare, 1972).

Whilst *T. evansi* causes disease in most domestic animals there is a considerable degree of variation in the severity of its pathological effects. This is probably due to variations in the virulence of a parasite strain and the degree of

susceptibility of the particular host animal. The clinical pattern of the disease is usually similar to nagana caused by *T. brucei* and is characterised by fever and anaemia, followed by oedema, cachexia and death. The disease usually assumes its most severe form in camels, horses and Asian elephants (Curasson, 1943). However, mild chronic infections which sometimes end in selfcure may occur in cattle, sheep and goats while in pigs, the disease, is as a rule, asymptomatic (Hornby, 1953; Mulligan, 1970).

The manner in which pathogenic trypanosomes produce disease and eventual death has been the subject of considerable research and controversy. It was believed that toxic substances detrimental to the host are liberated into the blood circulation from large numbers of dead and disintegrating trypanosomes. This, coupled with rapid exhaustion of blood glucose and uncompensated acidosis were thought to be central to the pathogenesis of trypanosome infections. However, Randall (1934) studied the blood chemistry of horses infected with *T. evansi* and noted that there was no evidence that the death of a large number of trypanosomes in the blood circulation liberated an endotoxin injurious to the animal. Also, studies of fluctuations in blood sugar levels in equines and camels infected with *T. evansi* showed that while blood sugar levels were lower at the time of crisis, they rose after the crisis and were at times higher than normal. In addition hypoglycaemia at time of death was not a marked feature (Randall, 1934). Conversely, it was demonstrated that experimentally infected animals were continuously in a state of acidosis which varied according to the number of trypanosomes in the blood, and that the end products of massive glucose consumption caused an uncompensated acidosis and exhaustion of alkali reserves which was further affected by the destruction of erythrocytes (Randall, 1934). This caused an anoxemia that manifested as accelerated and laboured respirations.

Surra is associated with changes in serum proteins in infected camels and buffaloes (Jatkar *et al.*, 1973; Verma and Gautam, 1977; Boid *et al.*, 1980). In experimentally and naturally infected Sudanese camels for instance, Boid *et al.* (1980) found that the total plasma protein concentration of infected animals increased above normal values although albumin levels decreased in both the natural and experimental infections. Although the significance of alterations in the levels of serum enzymes in the pathogenesis of *T. evansi* infections is not clear, it has been shown that in dogs and camels infected with *T. evansi* there is a significant increase in serum glutamate oxaloacetate transaminases (SGOT), glutamate pyruvate transaminase (SGPT), sorbitol dehydrogenase and alkaline phosphatase (Dwivedi *et al.*, 1977; Boid *et al.*, 1980).

In comparison to its tsetse-transmitted counterparts, the pathological

changes engendered by *T. evansi* in domestic animal hosts have received little attention from investigators (Stephen, 1986). Although leucocytosis is a consistent feature in naturally infected camels, decreases in erythrocyte counts, haemoglobin concentrations, lymphocyte and basophil counts have also been reported. However, there are concurrent increases in neutrophils, eosinophils and monocytes (Nadim and Soliman, 1967). Splenic and lymph node lymphoid hyperplasia with periportal accumulations of lymphohistiocytic cells in the liver has been seen in horses infected with *T. evansi* (Seiler *et al.*, 1981). In addition, scattered nonsuppurative myocarditis as well as evidence of haemosiderosis in phagocytic cells of the spleen, lymph nodes, liver and lungs has been observed. Histopathological studies of the central nervous system of infected horses showed a generalised, nonsuppurative meningo-encephalitis affecting the grey and white matter at all levels of the brain, with the severity of brain lesions varying from one animal to the other. Typically, broad perivascular cuffs of mononuclear inflammatory cells including large lymphocytes, histiocytes and some plasma cells were evident. Patchy gliosis which was often severe, accompanied the perivascular cuffing (Seiler *et al.*, 1981).

2A 2.4 Geographical Distribution of the Disease

The non-cyclical mode of transmission of *T. evansi* facilitated its dissemination throughout the tropical and sub-tropical regions of the world and the parasite has a very extensive geographical distribution. In the Old World, the disease is prevalent in countries lining the Atlantic and Mediterranean littorals such as Morocco, Algeria, Tunisia, Libya and Egypt, and extends south across the Sahara into Senegal, Mali, Chad and parts of the northern belts of West Africa from 13°-16°N. Eastwards the disease occurs in the Sudan to latitude 13°N and almost to the Equator in parts of northern Kenya and Somalia (Hoare, 1957).

The distribution of Surra on the Eurasian continent is rather uneven and patchy. It has been reported from Israel, Lebanon and Syria, the northern half of the Arabian Peninsula, Iraq and Iran. The trans-Volga region of the former Soviet Union is the area chiefly affected by 'Su-auru' in Europe, spanning areas from Kuibyshev (53°N) to the Caucasus (44°N). It then passes into Turkestan and extends into Burma, India, the Malay Peninsula, Indochina and parts of southern China (Hoare, 1972). The disease also occurs in Turkey and an isolated case has been recorded in Bulgaria. On the islands of the Indian Ocean the disease is prevalent in Mauritius and on the archipelagoes of Indonesia and the Philippines.

In the New World, *T. evansi* diseases known as Mal de caderas, Derrengadera and Murina occur in Central America and in parts of South America including Mexico, Venezuela, Columbia and Brazil (Hoare, 1972). Figure 2.4 shows

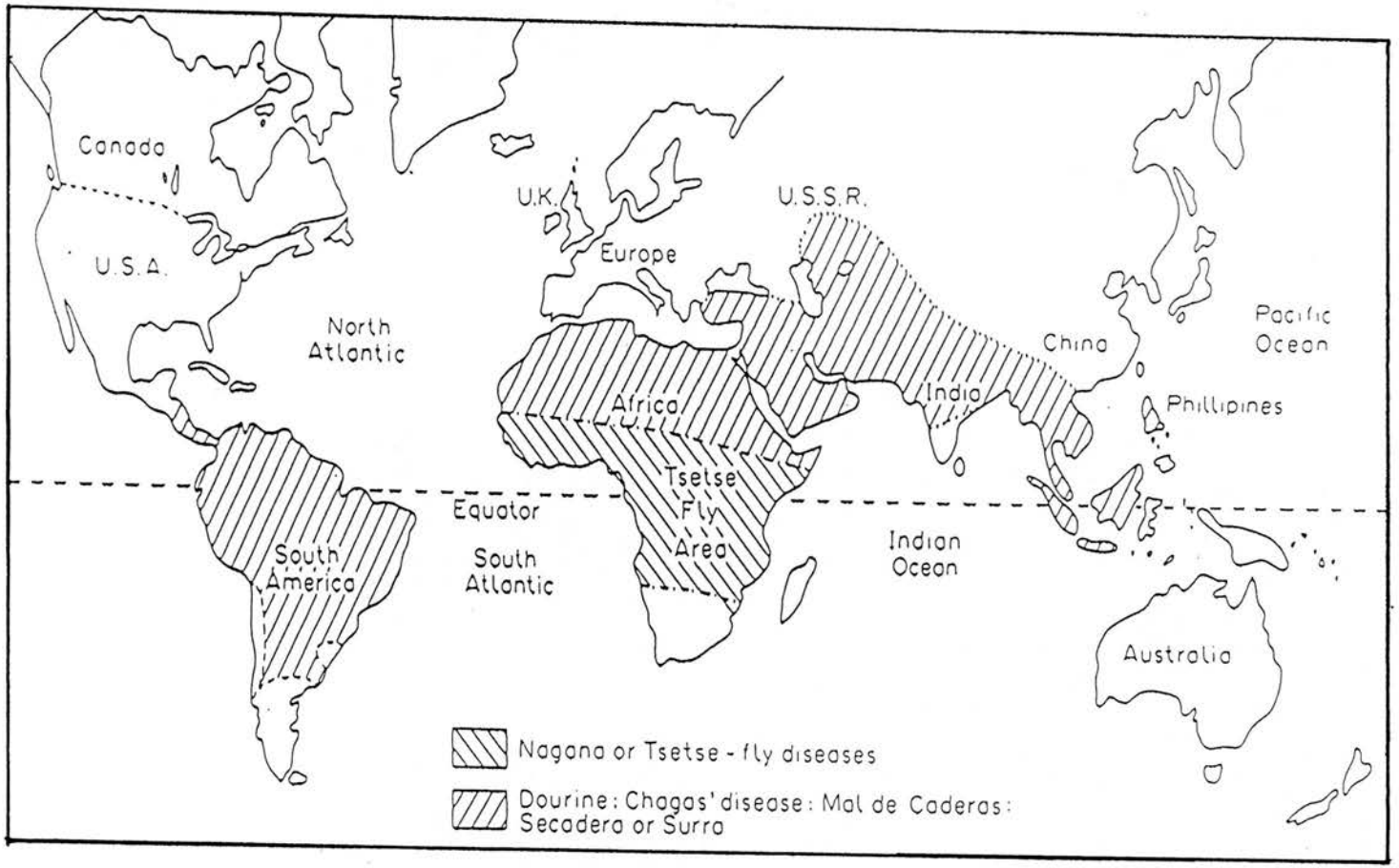


FIGURE 2.4 Geographical distribution—animal trypanosomiasis

the distribution of surra in the Old and New world.

2A 2.5 Antigenic Variation in African Trypanosomes

In the face of attack by specific and nonspecific immune systems, parasites use four evasion techniques to ensure survival: 1) sequestration in cells or body sanctuaries where immune attack is less effective, 2) deceit or mimicry by adsorption of host protein or immitation of host surface proteins, 3) rendering the attack futile by immune suppression and 4) antigenic variation, that is changing spontaneously the target of immune attack (Borst, 1991).

Antigenic variation is one of the most effective of the strategies developed by parasites to escape destruction. This strategy requires a large wardrobe of surface coats and mechanisms to exchange one coat for an unrelated one before the host's antibodies have destroyed the entire parasite population. No other parasite has developed and perfected this evasion strategy to the same high degree of sophistication as the pathogenic trypanosomes.

The success of pathogenic trypanosomes as parasites and the inability to develop an effective immunoprophylactic agent against them are largely as a result of their remarkable ability to undergo antigenic variation (Franke, 1905; Rodet and Vellet, 1906; Ritz, 1916; Leupold, 1928; Russell, 1936; Gray, 1962; 1965a; 1965b). The entire surface of the trypanosome, including the flagellum, is covered with a dense protein coat that consists of a single protein species known as the variant surface glycoprotein (VSG) (Vickerman, 1969; 1978; Cross, 1975; 1990). Antigenic variation in trypanosomes is a process whereby the parasites sequentially express and shed a series of different VSGs which then enables them to evade the host's protective immune responses. These surface antigens are capable of inducing protective immunity, and the immune response to each variant, although rapid and highly efficient in destroying any trypanosomes that possess that particular antigen, is invariably too late to affect trypanosomes which have altered their surface antigenic identity. Although antigenic variation in trypanosomes probably occurs when the organisms start dividing at the site of infection, in the chancre, it is in the systemic phase, as bloodstream forms, that the trypanosomes undergo extensive variation of surface antigens.

2A 2.5.1 The role of Antigenic Variation in the Establishment of Infection

The highly immunogenic VSGs determine the antigenic characteristics of the trypanosomes, known as variable antigen types (VATs) against which variant-specific immunity is induced (Barry and Turner, 1991). During an infection, although an immune response is elicited against a particular VAT, the trypanosomes

are able to express new and immunologically distinct VSG (Cross, 1978) which generates different VATs and results in the establishment of persistent chronic infection (Van Meirvenne *et al.*, 1975b). Clinically, this is manifested as fluctuating or successive waves of parasitaemia with the parasite staying one step ahead of the immune response. Each parasitaemic peak is a mixture of mainly predominant (homotype) VAT population and a small fraction of switched minor (heterotype) VAT population (McNeillage *et al.*, 1969; Van Meirvenne *et al.*, 1975a; Vickerman, 1978; Vickerman and Barry, 1982; Borst, 1991). The switching of the exposed variant-specific antigen in a subfraction of the parasite population and the expression of the surface antigen genes occur in a certain order which avoids simultaneous gross population heterogeneity. This in turn avoids rapid induction of antibodies against all members of the surface antigen repertoire which otherwise would ensure the ablation of the entire parasite population by the antibodies (Borst, 1991).

The total VAT repertoire and the genes encoding for them in a given clone of trypanosome is as yet undefined for any trypanosome species, but probably exceeds 100, as the VAT repertoire of a cloned isolate of *T. equiperdum* has been shown to do (Capbern *et al.*, 1977). However, to succeed as a means of self defence, ensuring establishment and prolongation of infection, the manner of expression of the VAT repertoire is perhaps more crucial to the trypanosome than the number of VATs generated. Thus, VATs from the repertoire must appear consecutively rather than simultaneously. If there were to be simultaneous expression of all VATs early in infection, it would dictate, either that the host becomes overwhelmed rapidly by fulminating parasitaemia or that effective immune responses would be generated against all VATs simultaneously. This does not happen and in fact, in bloodstream trypomastigotes, VATs appear in a certain hierarchical fashion. This hierarchy does not involve a simple, defined and nondiverging sequence of VAT expression. Such a simple, nondiverging sequence would reduce the establishment and longevity of infections initiated for instance, by trypanosome populations inoculated into partially immune reservoir hosts in the field. In such situations, the existing antibodies against individual VATs would simply terminate the sequence (Gray, 1965b; Capbern *et al.*, 1977; Miller and Turner, 1981; Barry and Turner, 1991).

VSG expression and antigenic variation, are features peculiar to bloodstream trypomastigotes. Following ingestion by *Glossina* there is cessation of this process during transformation from the bloodstream to the procyclic forms in the fly midgut (Ehlers, Czichos and Overath, 1987). In most salivarian trypanosomes, VSG is re-expressed in the infective metacyclic forms. In the case of *T. brucei* for instance, this starts in the metatrypanosomes in the tsetse salivary gland after the cessation of cyclical cell divisions (Tetley *et al.*, 1987). Upon

introduction into a new host, the manner of expression of the metacyclic VAT (mVAT) repertoire and their interaction with the host's initial immune responses in the chancre could determine whether an infection is established or aborted.

Although comprising only a limited proportion of the entire VAT repertoire of the bloodstream form, the metacyclic population is similarly antigenically heterogeneous and predictable (Le Ray, Barry and Vickerman, 1978). For example, there is limited mVAT repertoire in *T. rhodesiense* (Barry, Crowe and Vickerman, 1983; Turner *et al.*, 1988) and, in *T. congolense*, only 12 mVATs have been identified (Crowe *et al.*, 1983). The re-expression of mVATs following introduction into a host varies according to the species of trypanosome. For instance, mVATs of *T. brucei* are expressed only for a very short period following introduction into a new host (Barry *et al.*, 1983; Esser and Schrenbechler, 1985). In contrast, the mVAT of *T. congolense* continues to be expressed in chancres for up to six days after infection of rabbits and sheep (Luckins *et al.*, 1990; Sutherland, Ross and Luckins, 1991). The re-expression of mVATs is however, not restricted to infections initiated with metacyclic forms of trypanosomes but can also be seen later in infections initiated with bloodstream forms. This has been demonstrated for *T. rhodesiense* (Barry *et al.*, 1983), *T. congolense* (Nantulya *et al.*, 1984) and *T. vivax* (Nantulya, Musoke and Moloo, 1986), and indicates that despite antigenic variation, bloodstream trypanosomes are still able to re-express VATs which possess surface antigenic determinants identical to those of their metacyclic ancestors. This however, may not be to the advantage of the trypanosome, as such VATs would be easy prey to effector mechanisms which had been previously sensitised against them.

2A 2.5.2 The Variant Surface Glycoprotein

The VSG is a major cell product of the trypanosome and accounts for up to 10 percent of protein synthesised by the parasite (Cross, 1975; Bangs *et al.*, 1985; Ferguson *et al.*, 1986). It consists of a single polypeptide chain with an apparent molecular weight of 53 to 65 kD, but species differ widely in VSG isoelectric points, amino acid composition/sequence and carbohydrate content (Cross, 1975; 1977; Johnson and Cross, 1977; Hoeijmakers *et al.*, 1980b). The VSGs of *T. brucei* contain up to 470 amino acid residues (Allen, Dickens and Cross, 1982; Boothroyd *et al.*, 1982) in contrast to the 372 seen in *T. congolense* (Strickler *et al.*, 1987), while the carbohydrate component of various VSG species may vary from 7 to 17 percent (Johnson and Cross, 1977). Limited tryptic digestion cleaves all VSGs into two 'domains': an N-terminal (variable) fragment of MW 40-52 kD and a smaller C-terminal (constant) domain of MW 13 to 17 kD (Figure 2.5a; Johnson and Cross, 1979). There are no disulphide links between them and the entire molecule appears

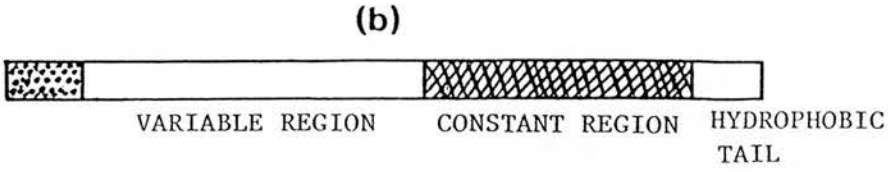


Fig 2.5 Line diagram of trypanosome VSG showing the hydrophobic tail

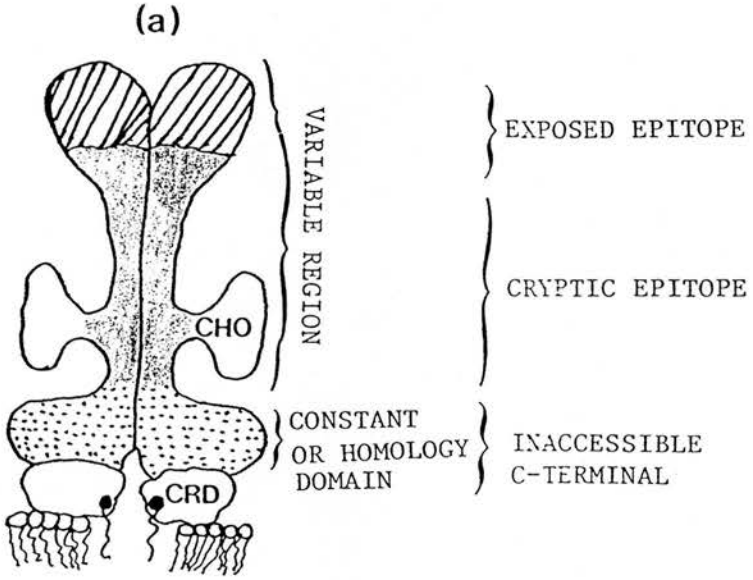


FIGURE 2.5 A dimeric unit model of trypanosome VSG based on EM and X-ray crystallographic studies (Cohen *et al.*, 1984; Freymann *et al.*, 1984; Metcalf *et al.*, 1987).

CHO = inaccessible oligosaccharide (carbohydrate) moieties linked to terminal amino acid asparginine.

CRD = cross reacting determinants which forms part of the membrane glycolipid attachment.

to be folded so that the N-terminal two-thirds and C-terminal third form the two distinct domains (Figure 2.5a).

Amino acid and nucleotide sequencing studies indicate that the immunological uniqueness of each glycoprotein resides in the variable N-terminal portion of the VSGs (Bridgen *et al.*, 1976; Rice-Ficht *et al.*, 1981; Miller *et al.*, 1984). Trypsinisation of living *T. brucei* releases the projecting N-terminus leaving the C-terminus (which is conserved) attached to the trypanosome membrane (Cross and Johnson, 1976). As shown by lectin binding studies on such live trypanosomes and by cytochemical staining as seen by EM, it appears that the oligosaccharide groups in *T. brucei* are located close to the membrane proper (Figure 2.5a; Wright and Hales, 1970; Steiger, 1973; Cross and Johnson, 1976; Seed *et al.*, 1976; Renwrantz and Schottelius, 1977). The inability of lectin to bind to the intact trypanosome suggests that the carbohydrate groups are inaccessible and therefore play no role in the antigenic specificity of the VSG molecule and thus no part in host-parasite interactions. It would therefore appear that cross reactivity between VSGs do not occur because of the hypervariability of the amino terminus. However, the work of Baltz *et al.* (1977) on lectin binding to *T. equiperdum* suggests that in the *brucei*-group there may be exceptions to this rule. In fact, although the amino acid sequence of the N-terminus confers great diversity of epitopes on each VSG molecule, some antigenic cross-reaction has been detected among various VSGs (Vickerman and Barry, 1982). Studies employing radioimmunoassay and microcomplement fixation tests have revealed cross-reacting groups which appear to be located in the C-terminus (Figure 2.5a) and involve carbohydrate moieties attached at, or very close to, the terminal amino acid, asparagine, aspartic acid or serine (Barbet and McGuire, 1978; Barbet *et al.*, 1979; Cross, 1979a; Holder and Cross, 1981). The conservation of sequence as shown by Rice-Ficht *et al.* (1981) within the C-terminal fifth of the protein would presumably also give rise to antigenic cross-reaction. Indeed, cross-reactions involving the entire VSG molecule have been known to occur in iso-VATs, which are common VSGs occurring in many serodemes as shown by competition radioimmunoassay, and by peptide mapping (Vervoort *et al.*, 1981). Serodemes are cloned stocks of a single trypanosome which express the same VAT repertoire (Anon, 1978).

It is not yet clear how the VSG is anchored to the membrane lipid bilayer although some affinity of purified VSG for cholesterol has been reported (Klein *et al.*, 1982). However, Johnson and Cross (1979) studying the soluble glycoprotein failed to demonstrate a hydrophobic segment which might serve to anchor the C-terminus to the membrane. Nevertheless, studies on the nucleotide sequence of VSG genes by recombinant DNA techniques, have suggested, that the primary translation product

contains a C-terminal hydrophobic 'tail' which is missing from the purified glycoprotein, (Figure 2.5a, b; Boothroyd *et al.*, 1980; Holder and Cross, 1981; Matthyssens *et al.*, 1981; Rice-Ficht *et al.*, 1981; Pays *et al.*, 1983). This loss may occur during intracellular processing but the tail may provide a VSG anchor in the intact cell and be shed only when VSG is released from the surface.

2A 2.5.3 The Mechanism of Antigenic Variation

Antigenic variation was originally conceived as an orderly programmed sequence of VATs, with VAT-specific host antibody probably acting as inducer of antigenic change in some of the trypanosomes before a relapse (Gray, 1965a). However, the detection of several heterotype VATs alongside the homotype, suggests that antigenic variation may be a spontaneous process, able to generate a large number of VATs in a given trypanosome population not necessarily in a programmed sequence. Moreover, host antibody response cannot be responsible for VSG switching since antigenic variation occurs in *T. brucei* and *T. congolense in vitro* (Doyle *et al.*, 1980; Luckins *et al.*, 1986) and in immunosuppressed infected animals (Mansfield, 1990). Host antibody pressure however, may be able to enhance antigenic variation by selecting for low numbers of new variant (heterotype) trypanosomes present in a population. In fact, the waning of antibody against a particular VAT might be expected to allow reappearance of that VAT in the same host. Such a situation has been reported in cattle infected with *T. brucei* (Nantulya *et al.*, 1979).

The rate and spontaneity of generation of heterotypes in a trypanosome population are suggestive of a background mutational mechanism for antigenic variation (Cantrell, 1958; Watkins, 1964; Seed and Gam, 1966). However, tendencies for variants of each strain of *T. brucei* to revert to a 'parent' antigenic type, and for variant antigens of cyclically transmitted strains to develop in an orderly sequence, argue against mutation as the basis of antigenic variation (Gray and Luckins, 1976). Moreover, the observed conservation of VAT repertoires and the VSG N-terminal amino acid analysis data demand that antigenic variation in the individual host has its basis in phenotypic rather than genotypic change (Vickerman and Barry, 1982).

It is now well established that sequential expression of a large series of different VSG genes is responsible for antigenic variation in pathogenic trypanosomes (Williams *et al.*, 1979; Hoeijmakers *et al.*, 1980a, b; Pays *et al.*, 1981). There is a separate gene for each VSG, which is present in each trypanosome of a serodeme regardless of whether the gene is being expressed or not. Estimates based on gene cloning and DNA hybridisation, suggest that each trypanosome contains

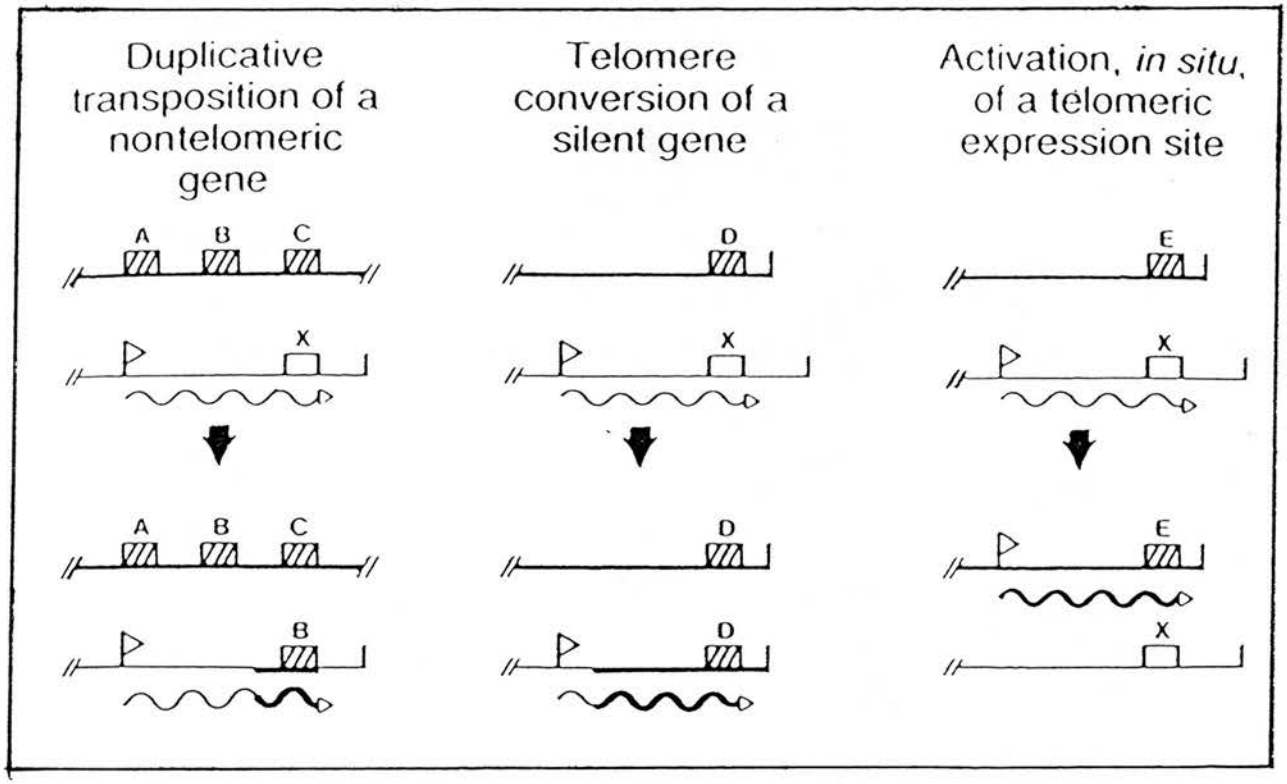


FIGURE 2.6 The various pathways for activating a variant-specific surface glycoprotein gene in *T. brucei*. 1) Boxes A, B and C are nontelomeric genes; X is a telomeric gene. Duplicative transposition of B displaces X and brings B under the expression site promoter (flag). The vertical bars mark the end of a chromosome. 2) Activation by duplicative transposition of the silent telomeric VSG gene, D, into a telomeric expression site. Because of considerable sequence homology between telomeres, large block sequence may be cotransposed, hence the name, telomere conversion. 3) Activation of an inactive telomeric expression site and concomitant inactivation of the previously active site. These two processes are spontaneous and independent of each other. Although gene rearrangements near the promoter are not involved, it is likely that further upstream, gene rearrangements may occur **(from Borst, P., 1991).**

1000-2000 VSG genes (Van der Ploeg *et al.*, 1982). The expression of a new VSG gene in some VATs is preceded by gene rearrangements in which formation of an expression-linked copy (ELC) from an existing basic copy of the VSG gene occurs. The ELC is subsequently transposed (Bernards *et al.*, 1981) to a telomeric expression site on one of the trypanosome's chromosomes, which contains an upstream promoter site (Figure 2.6). If a VSG gene already is in residence at the new telomeric transcription site, it is lost, degraded or inactivated when the new VSG gene is inserted into the telomeric expression site (Mansfield, 1990). Subsequently, the newly inserted VSG gene is transcribed, and the translational events ultimately result in the expression of a new VSG surface coat phenotype. In some variants, activation of a VSG gene that is already within a telomeric expression site occurs to produce a new surface coat. Also, a new VSG gene may be constructed not from a basic copy of the gene but through a recombinational event involving two different telomere-associated VSG genes (Borst, 1991).

The events regulating VSG genes within an expression site are not yet known (Borst and Cross, 1982; Mansfield, 1990) but as stated earlier, switching from one VSG to another is independent of antibody pressure (Doyle *et al.*, 1980; Luckins *et al.*, 1986). The pattern of antigenic variation at the cellular and molecular levels involving the frequency of switching between VSGs and VSG genes of a particular VAT population has been studied. By estimating the number of trypanosomes surviving antibody treatment it was shown that the number of switched trypanosomes ranged between 10^{-4} to 10^{-5} changes per cell per generation (Van Meirvenne *et al.*, 1975a; Doyle, 1977). However, since these figures only represent the number of switched trypanosomes in the population at a particular time, they cannot be judged to be accurate representatives of the frequency of VSG switching. A more accurate method would be estimates taking into account the growth rate of the newly expressed antigenic types, the proportion of switched trypanosomes and the number of generations from the original antigenically homogeneous population. Using this method, the frequency of switching from a defined VSG type to another was reported to range from 1.4×10^{-7} to 3.5×10^{-6} switches cell^{-1} generation^{-1} (Kosinski, 1980; Lamont, Tucker and Cross, 1986).

The genetic basis of antigenic variation, which leads to the switch in the VSG surface coat phenotype is thus the key to the success of trypanosomes as parasites. Since several copies of different VSG genes exist in a genetically homogenous trypanosome population (clone), the parasites are ensured of extended survival in an immunologically hostile environment. Certainly in nature, survival time of the parasite within the host is sufficient to result in transmission of the disease by the fly vectors before death of the host occurs.

It had been hoped that a multivalent VSG vaccine might be effective in controlling African trypanosomosis. However, this hope has been dashed because the sequence in VSG gene expression is virtually random, making it impossible to predict potential target antigen VSGs. The heterogeneity of VATs and the unpredictable VSG expression sequences in infected hosts therefore preclude development of multivalent VSG vaccines. Vaccination with the metacyclic trypomastigote VATs that are infected by tsetse flies is also problematic because similar metacyclic VSG gene heterogeneity and instability occur (Mansfield, 1990).

2A 3 Immune Responses and Immunity to Trypanosomes

The infection of humans, domestic livestock or laboratory animals with pathogenic African trypanosomes is invariably fatal if untreated as a result of the inability of the host to achieve a sterile immunity (Bancroft and Askonas, 1985). Nevertheless, it has been demonstrated experimentally that both domestic and laboratory animals can be rendered resistant to subsequent challenge with homologous trypanosomes by either needle or fly infection followed by drug treatment or by inoculation with irradiated trypanosomes (Whiteside, 1962; Wiesenhutter, 1970; Wilson *et al.*, 1975; 1976; Murray and Urquhart, 1977; Nantulya *et al.*, 1980; 1984; Welde *et al.*, 1981; Morrison *et al.*, 1982; Luckins, Rae and Gray, 1983). In naturally infected animals however, there is a wide variation in the immune response against trypanosomes. Thus, when cattle are infected with a single trypanosome serodeme they are sometimes able to eliminate the parasites and to recover (Morrison *et al.*, 1985). Even within the cattle population certain breeds such as the Muturu and N'dama indigenous to West and Central Africa have a greater capacity to control the level and duration of parasitaemia and thus survive (Murray, Morrison and Whitelaw, 1982). Immunity to trypanosomes is mediated by antibody responses against the VSG (Murray and Urquhart, 1977). The immunity is rapid and strong but because of antigenic variation is effective only against the VAT that elicited it (Vickerman and Barry, 1982; Morrison, *et al.*, 1985). A major feature of the immune response in trypanosome infections in domestic and laboratory animals is the alteration in cellularity and architecture of various lymphoid organs (Losos and Ikede, 1972; Mansfield, 1982; Bancroft and Askonas, 1985). In view of the importance of lymphocytes, their recirculation and the interaction between lymphoid cells, macrophages and other antigen presenting cells (APCs), it is to be expected that these changes would interfere with normal immune functions. Thus, one of the hallmarks of pathogenic African trypanosomes is the modulation of the host's immune system which results in generalised immunosuppression (Goodwin, 1970; Goodwin *et al.*, 1972; Murray *et al.*, 1974a).

Pathogenic trypanosomes are therefore able to elicit on one hand, responses which mediate protective (albeit nonsterile) immune response and on the other hand, those which modulate and compromise the immune competence of the host and ensure establishment and dissemination of the trypanosomes.

2A 3.1 Humoral Immune Responses to Trypanosomoses

The injection of trypanosomes by needle into a host triggers a rapid and strong antibody response. However, the typical primary humoral immune response of mammalian hosts, in which initial IgM synthesis is replaced by the synthesis of IgG antibodies, is somewhat modified in trypanosome infected hosts in that IgM production is greatly enhanced and prolonged. Serum hypergammaglobulinaemia of mainly the IgM isotype has been consistently found in trypanosome infections of laboratory rodents, humans, monkeys, bush buck, cattle and sheep (Houba *et al.*, 1969; Seed *et al.*, 1969; Luckins, 1972; 1975; Clarkson and Penhale, 1973; Greenwood, 1974; Hudson *et al.*, 1976; Mattern *et al.*, 1978; Mackenzie *et al.*, 1979). These elevations in IgM form the basis of a diagnostic test for human sleeping sickness. There is evidence that the increased level of immunoglobulins is not entirely parasite specific but consists of antibodies directed against both the trypanosomes and unrelated antigens as well as some autoantibodies. Thus, Houba *et al.* (1969) demonstrated the presence of heterophile antibodies in the serum of *T. gambiense*-infected monkeys. Much of the IgM is considered to be non-specific possibly resulting from polyclonal activation of B cells (Hudson *et al.*, 1976; Terry *et al.*, 1980). In mice infected with *T. brucei* for instance, less than 10 percent of the increased immunoglobulin produced is trypanosome specific (Corsini *et al.*, 1977). However, more recent work suggests that there is in fact a greater degree of parasite specific antibody since careful absorption with a wide range of trypanosome VATs can remove most of the IgM in serum of sleeping sickness patients (Herbert *et al.*, 1980) and *T. brucei*-infected cattle (Musoke *et al.*, 1981).

Despite conflicting views on the specificity of the IgM, there is ample evidence that the susceptibility or survival of trypanosome-infected hosts depends on their ability to mount an early IgM response. For instance, estimation of the immunoglobulin concentrations in C57B1 and A/J mice infected with *T. congolense* showed that the more resistant C57B1 strain developed a pronounced IgM response during the first parasitaemic wave. The more susceptible A/J mice not only failed in this but in addition, showed a fall in total IgG concentrations as well until death supervened 10 days after infection (MacAskill *et al.*, 1983). This result suggests that

the greater trypanoresistance of C57B1 mice largely depends upon its more efficient and earlier antibody responses (Whitelaw *et al.*, 1983). It is supported by the work of Mahan *et al.* (1986), who also showed that the ability of C57B1/6 mice to control the initial wave of parasitaemia following *T. vivax* infection and the failure of C3H/He mice to do so depended on the superior early and high levels of antibody production by the former mouse strain.

Trypanosome-specific antibodies are primarily directed against the VSG of the trypanosome surface coat (Black, Hewett and Sendashonga, 1982) but protection against the homologous clone can also be acquired by immunisation with the purified VSG (Cross, 1975). The primary antibody response of laboratory and domestic animals to trypanosome-bound VSG usually reaches a maximum 7-14 days after infection and then declines slowly, being detectable for more than 100 days (Seed *et al.*, 1969; Wilson and Cunningham, 1972; Herbert *et al.*, 1980; Pinder *et al.*, 1983). However, there appears to be a variation in the duration of the actual *in vivo* protection by these primary antibodies in animals, ranging in cattle from 35 days (Wiesenhutter, 1970) to 14 months (Wellde *et al.*, 1973). Similarly, the type of immunoglobulin produced during primary responses seems to vary. Hence, several workers using trypanosome agglutination test have reported that the primary response of rabbits to *T. gambiense* and rats or cattle to *T. brucei* is predominantly IgM (Seed *et al.*, 1969; Zahalsky and Weinberg, 1976). In addition, following primary challenge of mice with *T. gambiense*, purified IgM is 200-fold more efficient in a neutralisation of infectivity and 20-fold more effective in agglutination than IgG (Takayanagi and Enriquez, 1973). On the other hand, other investigators have shown that the primary antibody response of mice and cattle to *T. brucei* or mice to *T. rhodesiense* is predominantly IgG (Campbell, Esser and Phillips, 1978; Nantulya *et al.*, 1979; Sacks and Askonas, 1980; Mansfield *et al.*, 1981).

Few reports are available on the secondary antibody response to host reinfection with the same stock or clone of trypanosome. It appears, however, that the antibody kinetics, peak titre, and duration of detectable activity are similar to those after primary challenge in for example, rats infected with *T. brucei* (Zahalsky and Weinberg, 1976), mice inoculated with *T. gambiense* homogenate (Oka *et al.*, 1981), or in cattle infected with *T. brucei* (Pinder *et al.*, 1983). There is evidence that IgM antibody still predominates in secondary responses in cattle, although an appreciable amount of IgG is also produced (Roelants and Pinder, 1984). It was demonstrated by Musoke *et al.* (1981) that after both primary and secondary challenge VSG-specific IgM and IgG_1 but not IgG_2 were produced. In contrast, Pinder *et al.* (1983) observed both IgG_1 and IgG_2 during these challenges.

Cattle infected with *T. brucei* may, during the course of the infection, show

two peaks of antibody titre to the infecting organisms with the second peak possibly being higher than the first. These antibody peaks presumably arise because of the reappearance of organisms with the same or similar VSG (Nantulya *et al.*, 1979).

2A 3.2 Cellular Immune Responses in Trypanosomoses

Because of strong evidence that immunity to trypanosome infections is mediated by antibody, limited effort has been made to investigate cell-mediated immune (CMI) responses to trypanosomes. The result is that cell-mediated mechanisms directed specifically against trypanosome determinants and their relevance to protection are less well characterised than the humoral responses. However, the available evidence suggests that cell-mediated immunity against trypanosomes is either absent or relatively unimportant. Athymic nude mice and irradiated thymectomised mice are able to mount functional and in some cases enhanced immune response to *T. brucei* infection (Campbell *et al.*, 1978; Clayton *et al.*, 1979; Askonas *et al.*, 1979). In addition, immunity is not transferred adoptively with T cell-enriched spleen cell population (Takayanagi and Nakatake, 1975; Campbell and Phillips, 1976) and treatment of immune spleen cells *in vitro* with anti-thymic cell antiserum does not inhibit their capacity to transfer immunity (Takayanagi and Nakatake, 1975). In contrast, mice deprived of B cells are unable to mount an effective response (Campbell *et al.*, 1977) although immune mouse thymic cells can transfer immunity to thymectomised irradiated recipients (Tagayanagi and Nakatake, 1976).

It seems unlikely that effector T cells play a direct role in attacking the trypanosomes but there are significant changes in regulatory T cells and T cell functions which undoubtedly have a profound effect on immune function during infection. For instance, T cells from A/J mice primed by subcutaneous administration of live *T. brucei* (Gasbarre, Hug and Louis, 1980) or by immunisation with irradiated *T. rhodesiense* (Campbell *et al.*, 1978) will subsequently proliferate specifically *in vitro* in the presence of trypanosome antigens. This T cell proliferative response is however, abolished if active infection precedes immunisation by as few as 3 days (Gasbarre *et al.*, 1981). This suggests that T cell function is impaired by infection. Since the specific *in vitro* proliferation occurred after 5 and 10 days from the time of priming, and dropped or disappeared after 15 days, it was suggested that this was apparently as a result of T cell and macrophage unresponsiveness (Gasbarre *et al.*, 1981). However, the subcutaneous priming of mice is effective only when the live trypanosome is given with complete Freund's adjuvant (CFA). It is therefore difficult to assess the validity or importance of this *in vitro* T cell reactivity in relation to natural infections.

Studies of delayed type hypersensitivity (DTH) reaction have also been used to investigate T cell responses in rodent trypanosomosis. Tizard and Soltys (1971) found that rabbits infected with *T. brucei* or *T. rhodesiense* showed signs of DTH based on skin thickness and histologic examination of skin sections. Similarly, Finerty, Krehl and McKevin (1978) reported evidence of DTH in mice infected with *T. rhodesiense*, but the specificity for trypanosomes was not demonstrated. On the contrary, Mansfield and Kreier (1972) found hypersensitivity of the Arthus type in *T. congolense* infected and treated rabbits to intradermal challenge with sonically lysed homologous trypanosome antigen, but no evidence of DTH.

Similar observations of CMI responses have been made in trypanosome infected livestock. Emery *et al.* (1980) demonstrated that cattle which had been infected with *T. congolense* and treated with Berenil, developed characteristic DTH skin reaction when inoculated intradermally with ultrasonicated *T. congolense* antigen. Moreover, a marked proliferative response was obtained *in vitro* when PBLs from these animals were cultured in the presence of ultrasonicated homologous antigen. This PBL responsiveness was observed when the cells were collected from cattle infected with *T. congolense* either during the early stages of infection, before the development of chancre, or after the elimination of the parasites by chemotherapy (Emery *et al.*, 1980a). However, no response was detected in cattle with active infection,

Another aspect of cellular immune response to trypanosomes is the development of a skin reaction at the site of inoculation of the metatrypanosomes, which usually starts at about 5 to 7 days and peaks at about 9 to 12 days after the inoculation (Fairbairn and Godfrey, 1957; Bolton, 1965; Roberts, Gray and Gray, 1969; Gray and Luckins, 1980; Emery and Moloo, 1980; 1981; Akol and Murray, 1982; Dwinger *et al.*, 1987; 1988; 1989; Mwangi, Hopkins and Luckins, 1990). The histological appearance of the 'chancre', as it has been termed, has been described by different investigators as that of a polymorphonuclear, lymphocyte, lymphoblast, macrophage and plasma cell infiltration, (Emery *et al.*, 1980a; Gray and Luckins, 1980; Emery and Moloo, 1981; Akol and Murray, 1982; Morrison *et al.*, 1985; Mwangi, *et al.*, 1990; Taiwo *et al.*, 1990). The immunological role of the chancre is not clear but there are indications that trypanosome infected animals do not develop a chancre upon homologous (Emery *et al.*, 1980a; Mwangi *et al.*, 1990) or heterologous (Morrison *et al.*, 1982; Luckins and Gray, 1983; Dwinger *et al.*, 1989) challenge, while animals of low susceptibility develop smaller chancres. Furthermore, immunisation of goats with tsetse-transmitted *T. congolense* infection by drug treatment on day 13 p.i. after chancre development, gave rise to the development of protection to homologous challenge (Taiwo *et al.*, 1990). In contrast,

immunisation by intravenous inoculation of the metacyclics or treatment of the tsetse infected goats prior to development of chancre failed to induce such protection (Taiwo *et al.*, 1990). Thus chancre formation and the cellular changes which occur are vital for the induction of comprehensive immune recognition of metacyclic variable antigen repertoire deposited in the skin by infected tsetse, and hence the development of protective immunity.

2A 3.3 Induction of Protective Immunity Against Trypanosomes

Specific immunity, which results from specific recognition of antigen such that antibody or primed lymphocytes can react with that antigen, plays a significant role in determining the course and outcome of trypanosome infections. Numerous studies have shown that protective immunity can be induced in animals against specific populations of trypanosomes. The most commonly used methods for induction of immunity, as already mentioned, have been establishment of infection followed by chemotherapy or inoculation of irradiated non-infective trypanosomes.

Several early field studies with trypanocidal drugs showed that animals which had been treated were immune or partially immune to reinfection although the acquisition of immunity usually depended on the degree of trypanosome challenge and efficacy of drug treatment (Bevan, 1936; Whiteside, 1962; Wilson *et al.*, 1975; 1976; Leach and Roberts, 1981). Subsequently, it was demonstrated experimentally that cattle could be made immune to homologous challenge by a brief period of infection followed by trypanocidal drug treatment (Morrison *et al.*, 1985). Thus, Cunningham (1968) reported that 3 of 4 cattle which had been infected from a *T. brucei* stabilate and treated with Berenil were fully immune when challenged with the same stabilate 2 to 3 months after treatment. Wilson (1971) obtained similar results in five cattle infected with a *T. congolense* stabilate and treated with Berenil. However, Wellde *et al.* (1981) failed to induce protective immunity in nine cattle by treatment with Berenil at varying intervals after infection with *T. congolense* and challenge with the parent stock 28 to 128 weeks after treatment.

There is ample evidence that protective immunity induced against trypanosomes by infection and trypanocidal therapy is VAT specific and effective against VATs which develop during the course of the infection (Nantulya *et al.*, 1980; Luckins *et al.*, 1983; Akol and Murray, 1985). Mice primed by multiple bites from tsetse infected with a clone of *T. congolense* over an eight day period and treated 10 days after the first tsetse bite were fully immunised against the homologous clone when challenged on two occasions 21 days apart (Nantulya *et al.*, 1980). However, the immunity elicited in these mice was shortlived, lasting for only seven months.

Similar results were obtained by Luckins *et al.* (1983) in rabbits immunised by cyclical infection and treatment with homodium chloride seven days after the development of chancre. In this case, the immunity was of greater longevity, lasting for about 10 months. Studies by Akol and Murray (1985) showed that cattle infected with a cloned derivative of *T. congolense* and treated with Berenil 3 to 4 weeks later were immune to cyclical challenge with homologous clones 3 to 5 weeks later. However, immunity induced against stocks was not effective when the animals were challenged with the parent stock from which the clones were derived. These results were in agreement with those of Wellde *et al.* (1981) and emphasise the desirability of using cloned stocks in such studies. It indicates that the parent stock probably contained more than one serodeme, hence the immunity induced was specific only for the cloned VAT used. Similar results have also been obtained in goats infected with *T. brucei* (Emery *et al.*, 1980b), in sheep infected with *T. congolense* (Uilenberg, Maillot and Giret, 1973) and in cattle inoculated intradermally with cultured metacyclic forms of *T. congolense* (Akol and Murray, 1985).

Unlike infection and trypanocidal drug therapy, studies on induction of protective immunity by inoculation of irradiated noninfective trypanosomes indicated that large numbers of organisms were required to induce immunity. Thus, Duxbury *et al.* (1972) failed to immunise cattle against *T. congolense* following four to seven weekly doses of irradiated trypanosomes ranging from 10^8 to 10^9 organisms. When the animals were challenged with 10^4 to 10^5 trypanosomes, the only effect seen was an increase of about 3 days in the prepatent period. Using *T. brucei*, similar results were obtained in cattle by Duxbury *et al.* (1973). However, in further studies with *T. rhodesiense*, the same group was able to induce complete immunity in five cattle given six weekly i.v. inoculations of irradiated trypanosomes totalling from 3.5 to 6 x 10^{10} and challenged one week later (Wellde *et al.*, 1973). At face value therefore, it appears from these results that dead trypanosomes are not particularly immunogenic and that complete immunity against challenge with the homologous trypanosome population is not readily achieved. But a major flaw in all of these studies is the fact that they utilised uncharacterised stocks of trypanosomes. On the contrary, in a series of experiments examining the immunogenicity of either irradiated trypanosome or purified VSG from a well defined clone of *T. brucei* (ILT at 1.3), Morrison *et al.* (1982) found that a single i.v. inoculum of as few as 10^7 irradiated parasites in cattle conferred complete protection against challenge with 10^3 trypanosomes 14 days later. They further confirmed that immunity was mediated by antibodies to the VSG by immunising cattle with purified VSG. 200 µg of the purified VSG when administered on its own failed to immunise

the cattle but the same amount of VSG when incorporated in several different adjuvants resulted in immunity against challenge with 10^4 trypanosomes (Morrison *et al.*, 1982; Wells *et al.*, 1982).

Animals which recover spontaneously by self-cure also develop protective immunity to subsequent homologous challenge. Cattle which had undergone self-cure from a primary *T. congolense* infection after periods of 30-61 weeks of infection became fully immune when challenged with the same stock 25 to 54 weeks after parasitaemia had disappeared (Wellde *et al.*, 1981). Similarly, cattle experimentally infected i.v. with bloodstream forms of *T. brucei* and *T. congolense* self-cured 16 to 32 weeks after infection (Nantulya *et al.*, 1984). Sera from these animals contained neutralising antibodies against all mVATs of the infecting clones of *T. brucei* or *T. congolense*. All the animals were immune to challenge by bites of *G.m. morsitans* infected with the same clone or another clone of the same serodeme but they were susceptible to tsetse transmitted challenge with heterologous serodemes of *T. brucei* and *T. congolense*. It is interesting that sera from these animals contained neutralising antibodies to the mVATs of the infecting clones since it indicates that bloodstream trypanosomes are capable of expressing VATs which possess surface antigenic determinants identical to those of their metacyclic ancestor.

The relative ease with which protective immunity is induced against *T. brucei* and *T. congolense* contrasts with the situation in *T. vivax*. For instance, goats infected by tsetse-transmitted *T. vivax* and treated with a trypanocide failed to develop comprehensive immunity to homologous challenge (Vos, Moloo and Gardiner, 1988). Similarly, although cattle which had recovered from syringe-induced *T. vivax* infections developed resistance to subsequent tsetse-transmitted homologous challenge, Nantulya *et al.* (1986) failed to demonstrate neutralising antibodies to metacyclic forms of the *T. vivax* in serum of recovered animals at the time of challenge, suggesting that the immunity induced was specific for bloodstream rather than mVATs.

Most of the evidence for the role of antibodies in protective immunity during trypanosomosis derive from studies in small laboratory animals. However, there are now increasing data available from studies carried out in large livestock. *In vitro*, VAT-specific antibodies are known to bind readily to trypanosomes and cause various destructive effects such as agglutination (Cunningham and Vickerman, 1962), decrease in respiratory rate (Desowitz, 1956; Thurston, 1958), opsonisation (Lumsden and Herbert, 1967; Takayanagi *et al.*, 1974) and lysis (Lourie and O'Connor, 1936; Van Meirvenne *et al.*, 1975a). In mice, immunity can be transferred passively with serum from immunised animals (Seed, 1963; Watkins, 1964; Campbell and Phillips, 1976) as well as by adoptive transfer of spleen cells

from immunised to recipient mice (Luckins, 1972; Takayanagi and Nakatake, 1975; Campbell and Phillips, 1976). In large animals, there are major difficulties in studying the role of antibodies in protective immunity by carrying out passive or adoptive transfer experiments. However, Morrison *et al.* (1982) and Wells *et al* (1982) found a strong correlation between protection and the level of neutralising antibody in infected cattle.

Of all the methods used in inducing specific protective immunity, the use of intact trypanosomes (i.e. irradiated trypanosomes or live trypanosomes followed by treatment) is the more effective method. The level, specificity and class of antibody elicited by inoculation of irradiated *T. brucei* were found to be similar to those produced by infection with the living parasites followed by treatment (Morrison *et al.*, 1982). In both cases IgM and IgG antibodies were produced which were specific mainly for determinants on the surface of the live trypanosomes. This contrasts with results obtained with purified VSG which proved to be much less immunogenic unless it was potentiated by the use of adjuvants (Morrison *et al.*, 1982; Wells *et al.*, 1982). These results further emphasise the fact that immunological recongition of the VSG occurs mainly at the level of the intact trypanosome rather than the released soluble VSG.

As regards immune responses and induction of protective immunity to the non-tsetse-transmitted *T. evansi*, there are few studies of comparable detail. The presence of circulating antibodies in the sera of camels, goats and sheep infected with *T. evansi* has been demonstrated using passive haemaglutination, ELISA and complement fixation test (Schoening, 1924; Jatkar and Singh, 1971; Boid *et al.*, 1981). There is limited information on Ig class production in *T. evansi* infections but these indicate the predominance of IgM as seen in other trypanosome infections (Luckins, 1977; Luckins *et al.*, 1978; 1979; Boid *et al.*, 1985). In contrast, Uche (1989) using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and 'Western' blotting showed that although IgA, IgG and IgM were produced during primary and secondary experimental *T. evansi* infection in rabbits, IgG was the predominant antibody produced on both occasions. In camels experimentally infected with *T. evansi*, there were two-fold increases in serum IgM levels 22 to 29 days after infection which persisted for up to 42 days after treatment. In others, increase in IgM levels occurred 16 to 29 days after infection but the values fell below significant levels before treatment with quinapyramine (Luckins *et al.*, 1979). In naturally infected camels, IgM levels were low although the direct relationship between overt infection and the presence of antibody in the serum was good (Luckins *et al.*, 1979). Similarly, the levels of IgM in both naturally and experimentally infected camels increased significantly by as much as five times pre-

infection levels and remained high despite drug treatment (Boid, *et al.*, 1980). These workers found that IgG antibodies showed little significant change during infection.

There are few studies on the role of cellular immune responses in *T. evansi* infections. Gomez Rodriguez (1956) suggested that the cellular components of the host's immune system was important in the destruction of *T. evansi* and emphasised the importance of the phagocytic cells found within the leucocyte population, the liver and the spleen in the removal of the parasites. However, recent studies have shown that PBLs from *T. evansi*-infected and treated ponies showed strong *in vitro* responses to soluble homologous *T. evansi* antigen, but failed to respond to either the homologous live organisms or the purified VSG (Ahmed *et al.*, 1988). Upon stimulation, the cells were able to produce interleukin 2(IL-2) and express IL-2 receptor (IL-2R) (Ahmed, Schmid and Horchner, 1990). As in cattle infected with *T. congolense*, these responses were not observed in the ponies during active infection.

There is a dearth of information on the induction of protective immunity in *T. evansi*-infected animals. However, there is evidence of the existence of natural resistance to infection from both field observations and laboratory investigations. Field observations have shown that after recovery from infection, the host may acquire some immunity to reinfection with the homologous trypanosome while remaining susceptible to challenge with a heterologous one (Laveran and Mesnil, 1912: cited by Hoare, 1972). Bennet (1933) studied surra in camels in the Sudan and noted that recovery from the infection resulted in the development of specific immunity. Since the period of resistance to reinfection which he observed was of short duration, he concluded that in practice, immunity in camels must be considered as non existent. Development of resistance to *T. evansi* in the field also occurs following infection and treatment as is evidenced by the history of the disease in Mauritius (Adams, 1935). Following its initial introduction to the island in 1901, *T. evansi* caused devastating epizootic outbreaks which decimated the local cattle stock. But since 1921, when the disease was brought under control by a massive treatment campaign, the infection in bovines has assumed a mild latent form (Hoare, 1972). Experimentally, it has been demonstrated that rodents immunised with *T. evansi* are fully protected against challenge with homologous strains or serological variants but not against heterologous ones (Gill, 1965a, b, c).

2A 4 Immunosuppression and African Trypanosomoses

Pathogenic trypanosomes induce a generalised immunosuppression of humoral antibody responses as well as T cell-mediated immune responses both to the parasite and non-parasite antigens. As a result, in the long term, the host's immune responses to the parasite fail and it succumbs as a result either of overwhelming parasite loads, especially in rodents, or of secondary infection. Immunosuppression therefore has a three fold effect: (1) participates along with antigenic variation in ensuring the survival and dissemination of the parasites in the host, (2) predisposes hosts to secondary infections and (3) possibly compromises effective immune responses to vaccine antigens.

The earliest evidence of trypanosome-mediated immunosuppression are in reports by Parkin and Hornby (1930) who observed that cattle infected with *T. congolense* became incapable of thriving, lost condition and became susceptible to intercurrent infections. This observation was supported by Fiennes (1954) who reported enhanced susceptibility to bronchopneumonia in cattle suffering from trypanosomosis. More recently, conclusive support for the immunosuppressive effects of trypanosome infections have been obtained from numerous experiments carried out mainly in rodents, some of which are reviewed below.

2A 4.1 Immunosuppression in Infected Rodents

Experimental evidence that immunosuppression is a feature of trypanosomosis came from the observation that mice infected with *T. brucei* showed reduced antibody responses to sheep red blood cells, SRBC (Goodwin, 1970; Goodwin *et al.*, 1972). Subsequent corroboration of this work came from observations that trypanosome infections in mice were associated with profound suppression in antibody responses in response to challenge with a variety of antigens including T-dependent (SRBC) and T-independent (DNP-Ficoll) antigens as measured by serum antibody levels and numbers of splenic plaque-forming cells (PFC) (Longstaff, Freeman and Hudson, 1973; Murray *et al.*, 1974a; 1974b; Hudson *et al.*, 1976; Eardley and Jayawardena, 1977; Jayawardena *et al.*, 1978; Hudson and Terry, 1979; Wellhausen and Mansfield, 1979). Furthermore, spleen cells from *T. brucei* or *T. congolense* infected mice failed to respond *in vitro* to stimulation with either the T cell mitogens concanavalin A (Con A) and phytohaemagglutinin (PHA) or the B cell mitogen bacterial lipopolysaccharide (LPS). In addition, they suppressed the ability of normal mouse spleen cells to respond to the same mitogens (Albright, Albright and Dusanic, 1977; Jayawardena and Waksman, 1977; Corsini *et al.*, 1977; Pearson *et al.*, 1978; 1979; Roelants *et al.*, 1979a; Mansfield *et al*, 1981).

Throughout the course of infection, variant-specific antibodies continue to

be produced against successive VATS but there is evidence that the degree of antibody response to the parasite itself can also be depressed (Hudson and Terry, 1979). Mice infected with *T. brucei* exhibited depressed antibody responses as compared to uninfected controls, following inoculation of irradiated trypanosomes and the degree of the depression was related to the virulence of the infecting organisms (Sacks and Askonas, 1980). Furthermore, by examining the antibody responses of chronically infected mice to organisms derived from each of the first three waves of parasitaemia, it was shown that there was a progressive suppression of both IgM and IgG antibody responses to the parasites, such that by the third parasitaemic wave only low levels of IgM remained while there was no detectable IgG (Sacks and Askonas, 1980).

The process of immune expulsion of intestinal nematodes such as *Nippostrongylus brasiliensis* and *Trichuris muris* from both primarily infected and hyperimmune hosts is suppressed by *T. brucei* infections in mice and rats (Urquhart *et al.*, 1973; Phillips *et al.*, 1974). Rats infected with *N. brasiliensis* and then with *T. brucei* after the phase of immune expulsion of the worms, lose the ability to expel a challenge infection, but if the trypanosome infection is treated they can mount a full secondary response. This suggests that although trypanosome infection blocks the expression of a secondary immune response, immunological memory is preserved. It has been shown however, that both B and T cell memory may be exhausted in *T. brucei* infected mice, since transfer of sensitised spleen cells from such mice does not enable an irradiated recipient to mount a secondary immune response (Askonas *et al.*, 1979).

Cell-mediated immune responses are also suppressed in trypanosome-infected rodents. In chronically infected mice the T cell responses in the ear, induced by oxazolone treatment are depressed as determined by measurements of ear thickness (Urquhart *et al.*, 1973; Murray *et al.*, 1974c; Ackerman and Seed, 1976) and by incorporation of ^{125}I-labelled iododeoxyuridine in cells in local draining lymph nodes (Jennings *et al.*, 1974. Furthermore, Mansfield and Wallace (1974) demonstrated partial suppression of DTH skin reactions to purified protein derivative (PPD) of tuberculin in rabbits infected for 6 weeks with *T. congolense* while Allt *et al.* (1971) reported a reduction in the development of experimental allergic neuritis in *T. brucei* infected rabbits. However, a contradictory result to this was reported by Mackenzie, Sibley and White (1979). In addition, normal cell-mediated immune responses have also been observed in *T. brucei* infection although in some cases, suppression became evident only during the terminal stages of the infection (Freeman *et al.*, 1973).

2A 4.2 Mechanisms of Trypanosome-induced Immunosuppression in Rodents

Immunosuppression in trypanosome-infected rodents is well established but the mechanisms of its induction and its relation to the lymphoid organ changes and altered cellularity are not clear. A number of mechanisms have been proposed to account for the generalised immune dysfunction.

1. **Polyclonal B cell activation**

Polyclonal activation of B lymphocytes by a non-specific trypanosome derived mitogen (Esuruoso, 1976; Assoku and Tizard, 1978; Askonas *et al.*, 1979; Clayton *et al.*, 1979; Oka, 1986; Oka *et al.*, 1984) leading to clonal exhaustion of B cell potential and function (Freeman *et al.*, 1973; Urquhart *et al.*, 1973; Hudson *et al.*, 1976; Mansfield and Bagasra, 1978) is among the proposed mechanisms. This theory finds support from the observed maximal production of plasma cells in, and increased proliferation of, cultured spleen cells from *T. brucei* infected mice. *In vitro* cell proliferation is accompanied by production of high levels of IgM and IgG which starts immediately the cells are in culture but declines as the infection progresses, suggesting exhaustion of B lymphocytes (Corsini *et al.*, 1977). Furthermore, the presence of high IgM levels (Seed *et al.*, 1969; Hudson *et al*, 1976) and heterophile and autoantibodies (Houba *et al.*, 1969; reviewed in Parratt and Herbert, 1979) in the sera of trypanosome infected animals, accompanied by failure of trypanosomes to absorb most of the IgM, have all been regarded as evidence that B lymphocytes are being activated in a non-specific manner (Hudson *et al.*, 1976; Terry *et al.*, 1980).

2. **Suppressor T cells and Macrophages**

A second proposed mechanism is the generation of suppressor cells namely, macrophages and suppressor T cells. Spleen cells from *T. brucei*- or *T. congolense*-infected mice fail to respond *in vitro* to stimulation with either the T cell mitogens Con A and PHA or the B cell mitogen LPS and, suppress the ability of normal mouse spleen cells to respond to the same mitogens (Jayawardena and Waksman, 1977; Corsini *et al.*, 1977; Pearson *et al.*, 1978; 1979; Roelants *et al.*, 1979a; Mansfield *et al.*, 1981; Alcina and Fresno, 1985; Borowy *et al.*, 1990). In addition, they actively suppress the PFC response of normal spleen cells to SRBC and DNP-Ficoll (Eardley and Jayawardena, 1977; Jayawardena *et al.*, 1978; Wellhausen and Mansfield, 1979), fail to respond to allogeneic mixtures of cells differing at major (H-2) and minor (M1s) lymphocyte stimulation loci and do not stimulate allogeneic cells in mixed lymphocyte reaction (MLR) experiments (Askonas *et al.*, 1979; Pearson *et al.*, 1978; Roelants *et al.*, 1979a; 1979b; Grosskinsky and Askonas, 1981). Since the removal of glass-adherent cells and treatment with anti-Thy 1 serum and complement restored the responses, it was concluded that immunosuppression is mediated by macrophages and suppressor T cells. It was

hypothesised that suppressor T cells directly stimulated by trypanosomes release factors having affinity for macrophages which in turn are activated to become suppressors for T and B cell responses (Eardley and Jayawardena, 1977; Roelants and Pinder, 1984). Alcina and Fresno (1985) proposed an alternative hypothesis, suggesting that suppression is a result of complex immunoregulatory circuit in which macrophages, after interaction with parasites, are able to induce the release of suppressor molecules that act preferentially on immune T cells, which in turn inhibit the proliferation of helper T cells by either defective IL-2 production or inhibition of IL-2 action or both. Although the nature of the suppressor molecules was not known, evidence in support of Alcina and Fresno's proposed central role for macrophages and the subsequent sequence of events culminating in T and B cell dysfunction is mounting (see below).

3. **The Role of Secretory Products of Macrophages and T Cells**

Recent investigations have focussed on impairment of secretion of various lymphokines by macrophages and T cells. Production of IL-2, a lymphokine which plays a central role in T cell activation and proliferation, from Con A stimulated splenocytes is suppressed in both susceptible (A/J) and resistant (C57BL/6J) inbred mice infected with *T. congolense* (Mitchell, Pearson and Gauldie, 1986). This is in agreement with work by Alcina and Fresno (1985) in which Con A-stimulated splenocytes from *T. brucei*-infected BALB/C mice failed to produce detectable amounts of IL-2. Similarly, the production of IL-2 by T cells was severely suppressed in Con A-stimulated spleen and lymph node cells from *T. brucei*-infected C3H/H3, CBA/Ca and (C57/BL/6xBALB/C)F_1 mice (Sileghem, Hamers and De Baetselier, 1986; 1987b; Sileghem *et al.*, 1987a). Furthermore, several groups exploring the mechanisms of immunosuppression in the related human Chagas' disease, caused by *T. cruzi*, have also demonstrated suppressed production of IL-2 by mitogen-stimulated spleen cells in infected rodents (Harel-Bellan *et al.*, 1983; 1985; Reed, Inverso and Roters, 1984a; 1984b; Tarleton and Kuhn, 1984; Choromanski and Kuhn, 1985; Tarleton, 1988a; 1988b). Recent reports have in addition identified diminished interleukin-2 receptor (IL-2R) expression by lymphocytes from mice infected with *T. brucei* (Sileghem *et al.*, 1987b; 1989a; 1989b; 1991) and *T. cruzi* (Rottenberg *et al.*, 1989). IL-2R of lymphocytes is a complex heterodimer which binds IL-2 with high affinity and is responsible for IL-2 internalisation as well as transduction of the signal required for activated lymphocytes to progress through their division cycle (Kierszenbaum and Sztein, 1990). Furthermore, it has been shown that there is elevated secretion of another cytokine, gamma interferon (IFN-γ) in both *T. brucei*- and *T. cruzi*-infected mice (Sileghem, Darji and De Baetselier, 1991; Nabors and Tarleton, 1991) and that this

elevation is responsible for the hyporesponsiveness of T cells (Darji *et al.*, 1991a). Based on these results, Sileghem *et al.* (1989a; 1989b; 1991) and Darji *et al.* (1991a; 1991b) argued that macrophages play the key role in T cell unresponsiveness and the subsequent generalised immunosuppression seen in murine trypanosomosis. They proposed that macrophages act through two different suppressive mechanisms; firstly, a prostaglandin (PG)-dependent mechanism which blocks the IL-2 secretion by T cells and secondly, a PG-independent mechanism which interferes at the level of IL-2R expression on T cells. In the PG-dependent mechanism, macrophages interact with a distinct trypanosomal fragment (of 70 kD MW) and become triggered to secrete PG. The secreted PG actively inhibits IL-2 secretion by $CD4^+$ T cells. In the PG-independent mechanism, the trypanosome triggered macrophages, by direct cell-cell contact, provide a costimulatory signal to T cells which in turn become triggered by exogenous stimuli (mitogens or antigens) to produce substantial amounts of IFN-γ. The IFN-γ produced by T cells and soluble factors released by the trypanosome-activated macrophages act in concert to inhibit IL-2R expression on $CD4^+$ and $CD8^+$ T cells. Furthermore, since IFN-γ is a potent macrophage activator, it may act through its macrophage activating property to fuel the suppression by further PG release which in turn inhibits IL-2 secretion by $CD4^+$ T cells.

4. **Defective Antigen Handling and Presentation**

Immunosuppression in rodent trypanosomosis is also thought to be mediated through defective processing of antigens by APCs and their inadequate presentation with MHC class II products (Mansfield and Bagasra, 1978). The progressive loss of the antigen-presenting (Ia antigen-bearing) macrophage subpopulation from the spleen, lymph nodes and peritoneal cavity of *T. rhodesiense*-infected mice (Bagasra, Schell and Le Frock, 1981) and the reduced expression of mannose, Fc-, and complement-receptors (Grosskinsky *et al.*, 1983) may result in an early reduction in antigen handling and presentation by macrophages during the disease.

2A 4.3 Immunosuppression in infected livestock

Efforts to demonstrate immunosuppression in domestic livestock experimentally infected with trypanosomes have been concentrated mainly on cattle. Available literature indicates that trypanosome-induced immunosuppression in these animals may not be as severe as in rodents since the disruption of the lymphoid architecture is less dramatic and parasitaemias seldom reach the fulminating proportions characteristic of rodent infections (Morrison and Murray, 1979; Masake and Morrison, 1981). The results of attempts to evaluate the immune

competence of trypanosome-infected ruminants in regard to their ability to produce antibodies to various bacterial and viral vaccine antigens have been inconsistent and conflicting.

There is depressed secondary antibody response to polyvalent clostridial vaccine in cattle infected with *T. congolense* (Holmes *et al.*, 1974). These authors drew attention to the potential danger of trypanosomal immunosuppression hampering the success of vaccination programmes carried out in endemic areas. Similarly, Scott *et al.* (1977) reported depression of serum antibody titres to the same *Clostridium oedematiens* Type B component of a polyvalent clostridial vaccine in cattle naturally and experimentally infected with *T. congolense*. However, antitoxin antibody titres were above the required minimum protective level and they concluded that whilst there was distinct evidence of trypanosome-induced immunosuppression to clostridial vaccines it was unlikely to interfere with regular vaccination programmes. They did not challenge the infected/vaccinated animals with the clostridial organisms in order to substantiate this conclusion. *T. vivax* infection alone or a combination of *T. congolense* and *T. vivax* infection, caused only slight depression of primary and secondary antibody responses in cattle vaccinated 6 and 18 weeks after infection with contagious bovine pleuropneumonia (CBPP) vaccine (Ilemobade *et al.*, 1982). In spite of the slight nature of the depressed antibody response, 50 percent of the infected/vaccinated cattle contracted CBPP following challenge with *Mycoplasma mycoides*. Cattle experimentally infected with *T. congolense* or *T. vivax* also showed slight suppression of antibody responses to *Brucella abortus* (S19), *Leptospira biflexa* and *M. mycoides* vaccine (Rurangirwa *et al.*, 1978, 1979; 1980). In a later study (Rurangirwa *et al.*, 1983) profound suppression of IgG_1, IgG_2 and IgM responses to *B. abortus* (S-19) vaccine was observed in cattle chronically infected with *T. congolense*.

Results of studies with viral vaccine antigens have also been inconsistent. For instance, whilst suppression of antibody response to louping ill virus vaccine occurred in cattle infected with *T. congolense* or *T. vivax* (Whitelaw *et al.*, 1979) there was no evidence for suppression of antibody response in cattle infected with *T. brucei*. Only slight suppression of antibody response to rinderpest live, attenuated vaccine was observed in cattle infected with *T. congolense* and *T. vivax* (Rurangirwa *et al.*, 1980). This inconsistent demonstration of immuno- suppression in trypanosome-infected livestock may be related to the fact that most of the studies have concentrated on the first few weeks of the infection and it is also possible that infections of longer duration are more likely to result in functionally significant immunosuppression as demonstrated by Rurangirwa *et al.* (1983).

Studies on trypanosome-induced suppression of cellular immune

responses are equally limited and conflicting. Using cell reactivity in mixed-lymphocyte cultures (MLC) and mitogen-induced lymphocyte transformation *in vitro* to study T cell responses in *T. vivax*-infected goats, Van Dam *et al.* (1981) claimed that *T. vivax* infection in goats was associated with severe (50-100 percent) suppression of mitogen-induced lymphocyte function. This suppression became apparent from day 20 after the infection. Similarly, the reactivity of lymphocytes from infected goats in MLC was diminished and remained so for up to 2 weeks after Berenil treatment when restoration of functional capacity appeared. In contrast, Sollod and Frank (1979), Masake and Morrison (1981) and Masake *et al.* (1981) failed to demonstrate significant suppression of the *in vitro* responses to mitogens of leucocytes from cattle infected with *T. congolense* or *T. vivax* . Furthermore, there was no significant suppression of the responses in mixed lymphocyte reaction (MLR) of PBL, lymph node and spleen cells of cattle infected with *T. congolense* (Morrison *et al.*, 1985).

The suppression of specific antibody or cellular responses to the trypanosomes themselves has also been investigated. The antibody response of cattle was examined following simultaneous or sequential challenge with two clones of *T. brucei.* It was reported that the response to one of the clones was partially depressed in animals inoculated with both clones either simultaneously or 2 days apart (Nantulya *et al.*, 1982). Similarly, Morrison *et al.* (1985) examined the response of cattle infected with *T. congolense* to fixed numbers of an irradiated clone of *T. brucei* which was inoculated either intravenously or by the subcutaneous route. It was found that 60 and 40 percent respectively of cattle inoculated with the irradiated *T. brucei* showed marked suppression in antibody response as measured by the Farr assay with purified VSG. The degree of suppression depended on the severity of the *T. congolense* infection. In studying the cellular responses to specific trypanosomal antigen it was demonstrated that PBL from infected and treated cattle, proliferated *in vitro* in response to stimulation with homologous, ultrasonicated trypanosomal antigen (Emery, Wells and Tenywa, 1980). However, this response was completely suppressed in animals with active infections. On the other hand, in cattle cyclically infected with *T. congolense*, PBL collected between days 3 and 7 post infection (i.e. before chancre development) showed a strong proliferative response to ultrasonicated trypanosomal antigen but thereafter, the response was suppressed reappearing only after the animals had been treated (Morrison *et al.*, 1985). Hence, while T cell responses are suppressed during infection in large animals, immunological memory is not affected.

2A 4.4 Mechanisms of Trypanosome-induced Immunosuppression in Large

Animals

The mechanisms of trypanosome-mediated immunosuppression in domestic livestock is ill-understood. The subject has not been studied on the same scale as in rodents probably as a result of the expense of the acquisition and management of large animals as experimental models. It is possible that some of the mechanisms suggested in rodent trypanosomosis such as polyclonal B cell activation, generation of suppressor T cells and macrophages may operate in trypanosome-infected livestock. Hypocomplementaemia and increased immunoglobulin catabolism occur in cattle infected with *T. congolense* which could be responsible for the depressed antibody levels in infected animals (Nielsen *et al.*, 1978a; 1978b). *In vitro* proliferative responses of PBLs from animals harbouring active infection is suppressed following stimulation with parasite antigens or mitogens. This suggests that, as in infected rodents, T and B cell responses are also depressed during infection. It is doubtful however, whether polyclonal B cell activation could account for the loss of B cell potential and responsiveness in ruminants. Although hypergammaglobulinaemia involving mainly IgM is a consistent feature of livestock trypanosomoses (Clarkson *et al.*, 1975; Luckins and Mehlitz, 1976; Kobayashi and Tizard, 1976; Nielsen *et al.*, 1978b; Masake and Morrison, 1981), increase in the levels of heterophile antibodies has not been reported. The mechanisms of trypanosome-mediated immunosuppression in large animals may not parallel those operating in rodents, as illustrated in the case of *T. cruzi* infections in rodents and in man. *T. cruzi* induces severe immunosuppression in both naturally infected human patients and in experimentally infected rodents (Clinton *et al.*, 1975; Reed, Larson and Speer, 1977; Teixeira *et al.*, 1978; Kierszenbaum, 1981; Voltarelli *et al.*, 1987), but it has been shown that the mechanisms of the induction of the suppression are not entirely similar in both cases. For instance, parallel studies conducted with normal murine splenocytes and human peripheral blood mononuclear cells co-cultured with *T. cruzi* trypomastigotes, revealed that the capacity of mouse cells, but not the human cells, to secrete IL-2 in response to mitogen stimulation is suppressed (Beltz, Sztein and Kierszenbaum, 1988). Furthermore, only mouse lymphocyte proliferative responsiveness is restored by the introduction of exogenous IL-2 (Beltz *et al.*, 1988). Production of IFN-γ by mitogen-stimulated lymphocytes is suppressed by co-culture of the mouse, but not human, lymphocytes with *T. cruzi* (Beltz, Sonnenfeld and Kierszenbaum, 1989). Unlike the mouse model system, *T. cruzi* induced suppression of human PBMC occurs in the face of normal IL-2 and IFN-γ production, suggesting a role for T_H2-like cells in the suppression of human PBMC responses (Beltz and Kierszenbaum, 1987; Beltz *et al.*, 1989). *T. cruzi* selectively

exerts its suppressive effects on human lymphocytes through the inhibition of IL-2R and transferrin receptor (TfR) expression on $CD4^+$ and $CD8^+$ T cells and through reductions in the numbers of cells expressing CD3, CD4 and CD8 molecules which play key roles in lymphocyte activation (Beltz *et al.*, 1988; 1990; Kierszenbaum and Sztein, 1990; Sztein, Cuna and Kierszenbaum, 1990).

Similar differences may exist in the mechanisms of immunosuppression seen in infected rodents and large animal hosts. There is virtually no information on the role of suppressor T cells and macrophages in the immunopathology of trypanosome infections in domestic livestock. However, there is evidence that in cattle and sheep infected with *T. congolense*, major alterations occur in various lymphocyte surface molecules which play major roles in effective immunity and that aberrations such as large increase in $CD5^+$B cells is a feature (Ellis *et al* ., 1987; Mwangi, Hopkins and Luckins, 1990; 1991; Mwangi, 1991; Williams *et al.*, 1991). These changes in the lymphocyte subsets, some of which correlate with resistance and self-cure (Williams *et al.*, 1991), may underlie the immunosuppression that occurs in livestock trypanosomosis. However, their further and extended analysis over time and in relation to mitogenic and specific antigenic responses is still required before their role in the immunosuppressive effects of trypanosomes can be fully determined.

SECTION B

Immunobiology of the Sheep Lymphoid System

2B 1 Lymphocyte migration in sheep

Although lymphoid organs are dispersed all over the body, they are connected by two vascular networks: the blood vessels and the lymphatic ducts, which enables the lymphoid organs to function as a single system. The major cellular constituent of this system namely, lymphocytes, are mobile, travelling from blood to lymph and then back to the blood (Gowans, 1957; Gowans and Knight, 1964). This orderly movement of cells between the various lymphoid compartments ensures a coordinated expression of immunological functions by the system. Lymphocytes are the major cellular participants in immune responses and it appears that the reason for their recirculation is purely immunological. This is because recirculation ensures that: 1) the efficiency of regional immune responses is optimised by allowing antigen-induced selection of precursor cells from a larger pool than is present in regional lymph nodes alone (Gowans and Steer, 1980); 2) enables systemic immunity to be induced by local entry of antigen due to the dissemination of effector and memory cells (Smith *et al.*, 1970); 3) facilitates the collaboration of T and B lymphocytes in immune responses by enabling antigen-sensitive cells of each type to come together (Ford, 1975); 4) achieves control of immune responses by the recruitment and transportation of regulatory cells (Gowans, 1980) and finally, 5) gives antigen-sensitive cells an opportunity to dissociate themselves from a particular environment from time to time so that not all of them are driven to the stage of terminal differentiation (Morris, 1980). Since the ovine placenta is syndesmochorial in nature, the ovine foetal immune system develops in an environment devoid of extrinsic antigens, primed lymphocytes and circulating antibodies. However, lymphocyte recirculation still takes place actively in ovine embryos (Pearson *et al.*, 1976). Thus, although there are immunological reasons for lymphocyte recirculation, the capacity to do so also arises as a natural physiological process independent of prior stimulation with external antigens.

Large numbers of lymphocytes continually leave the circulation through specialised post-capillary venules in the cortex of the lymph node and enter the efferent lymph (Gowans and Knight, 1964; Hall and Morris, 1965). This migration of lymphocytes between the blood and lymph is non-random (Scollay, Hopkins and Hall, 1976; Cahill *et al.*, 1977; Issekutz, Chin and Hay, 1982). Their exit from the blood is controlled by specific molecular interactions between lymphocytes and endothelial cells, and tissue-specific migration-related receptors directing lymphocytes to gut and peripheral lymph nodes (Gallatin, Weissman and Butcher, 1983; Woodruff, Clarke and Chin, 1987; Streeter *et al.*, 1988).

The recirculation of lymphocytes, especially the examination of cells migrating in afferent and efferent lymph, has been extensively studied in sheep

(Smith, McIntosh and Morris, 1970; Morris and Courtice, 1977; Mackay *et al.*, 1988a; Washington, Kimpton and Cahill, 1988). There is no doubt that the recirculation of cells provides the means whereby large numbers of lymphocytes with different antigen specificities may encounter antigen, allowing specific antigen-reactive cells to be selected and to accumulate at sites of antigen deposition. There is now a wide body of knowledge on immune and inflammatory responses to introduced antigens or pyrogens and the modulatory effects of antigens upon the migratory pathways of lymphocyte subpopulations between the vascular and lymphoid compartments (Reviewed by Hall and Cahill, 1982). Cannulation of afferent and efferent lymphatic ducts of lymph nodes have enabled the study of the cell kinetics in the immune response to percutaneous or intralymphatic infusions (Hall and Morris, 1962; 1965; Hay, Cahill and Trnka, 1974) and in the responses to grafted tissues and organs (Hall, 1967; Pederson and Morris, 1970). The development of hybridoma technology and mAbs to surface antigens of sheep lymphocytes and other leucocytes has allowed the extension of these studies of lymphocyte migration in sheep into precise definition of the qualitative and quantitative cellular kinetics of lymphocyte phenotypes in the immune response and their functional roles in immunity and pathogenesis of disease (Mackay *et al.*, 1985; Puri, Mackay and Brandon, 1985; Hopkins *et al.*, 1986; 1989; Mackay *et al.*, 1986; 1987; 1988a; Kimpton, Washington and Cahill, 1989).

2B 1.1 Cell Migration From Areas of Antigen Deposition in the Skin

The skin contains small numbers of lymphocyte and macrophages which migrate into the draining lymph nodes via the dermal lymphatic network which anastomoses to form larger afferent collecting lymphatics. These lymphatics have few open junctions in addition to oneway valves and smooth muscles which by spontaneous contractions propel lymph towards the lymph node. The ablation of peripheral lymph nodes is followed by anastomosis of the afferent ducts with the efferent duct (Trevella and Morris, 1980) which has allowed the study of cells migrating in cutaneous lymph by cannulating the resulting pseudoafferent duct (Hopkins *et al.*, 1985). Most of the cells in lymph draining subcutaneous tissues in sheep are mononuclear cells in which lymphocytes predominate. Also present is a small proportion of cells of the monocyte/macrophage lineage (Miller and Adams, 1977) and microphage-like cells having cytoplasmic veils (Morris, 1972) which are in close association with lymphocytes. Following the injection of foreign antigen into the skin, there is alteration in the composition of the lymph draining the area such that it contains granulocytes, erythrocytes and antigen-antibody complexes (Hall and Morris, 1963; Hay and Cahill, 1982; Hein *et al.*, 1987). In addition, the

lymphocyte output increases as has been shown in afferent lymph draining granulomatous and DTH lesions (Smith *et al.*, 1970; Hay, Lachman and Trnka, 1973) and in sheep infected with *T. congolense* (Mwangi, 1991). The neutrophil output is also increased and this is a characteristic feature of acute inflammatory reactions elicited by skin contact with DNFB (Hall and Smith, 1971). Alterations in the cellular composition of lymph draining areas of antigenic stimulation in the skin are also accompanied by dramatic changes in the expression and output of various lymphocyte phenotypes. For instance, following *in vivo* secondary antigenic stimulation using OVA, PPD or sperm whale myoglobin, in the draining area of the afferent lymphatic, the proportion of MHC class II-positive cells increase nearly three-fold (Hopkins *et al.*, 1989). Following intradermal infection of sheep with cultured metacyclics of *T. congolense*, the proportions of $CD5^+$ and $CD4^+$ cells markedly increase after the onset of parasitosis but later decline as the infection progressed (Mwangi, 1991).

2B 1.2 Cell Migration and Localisation in Antigen-stimulated Lymph nodes

Lymph nodes are encapsulated secondary lymphoid tissues which act as filters for foreign materials which enter the peripheral lymphatics and provide a favourable environment for the interaction of different cell types in the generation of immune responses to lymph-born antigens. Following antigen stimulation of the skin, lymphoproliferation and increased recruitment of cells from blood and lymph occur, resulting in the gross enlargement of the lymph node. T-dependent antigens induce active T cell proliferation in the paracortical areas while stimulation by T-independent antigens results in a greater proliferation of B cells in the cortex. However, due to the heterogeneity of most antigens, the response seen is usually that of generalised hyperplasia of both T and B cell areas. Germinal centres and secondary follicles contain actively proliferating B cells while secondary follicles contain dendritic cells, macrophages and few T cells (Mwangi *et al.*, 1991). Together with the specialised macrophages of the marginal sinus, these cells play a role in B cell responses and the development of B memory cells.

2B 1.3 Cell Constituents of the Efferent Lymph

Lymphocytes are the predominant cellular constituents of efferent lymph and large numbers exit the nodes. From a one-gram popliteal lymph node of sheep that contains approximately 10^9 lymphocytes, about 3×10^7 of them leave via efferent lymph every hour, whereas only less than 10 percent of that number enter the node via afferent ducts. Local irradiation of a single lymph node does not result in the obliteration of the cellular output, suggesting that the majority of efferent

lymph lymphocytes are not generated within the node but are derived from the blood perfusing the node (Hall and Morris, 1964). This finds support in studies which showed unequivocally that during continuous infusion of ^{3}H-thymidine(^{3}H-TdR) into a sheep popliteal lymphnode through an indwelling catheter in an afferent lymphatic, less than 4 percent of the cells found in efferent lymph could be labelled by ^{3}H-TdR infusion throughout the whole course of the experiment. Clearly, this is an indication that the majority of efferent lymph lymphocytes were not derived from the node but were blood-borne (Hall and Morris, 1965).

Studies on the response of a single lymph node to antigenic stimulation revealed that dramatic changes occur in the cell content of efferent lymph. In the first 24 hours after antigenic challenge, cell traffic is greatly reduced (Hall and Morris, 1965). Subsequently, cells are recruited from the blood into the node where they undergo a process of selection, proliferation and differentiation into antibody synthesising cells. At 72 to 96 hours after challenge, the cell output increases to a maximum of 5-10 times above prestimulation levels and large blast cells and antibody forming cells appear (Fahy *et al.*, 1980). The cells leaving the node disseminate the immune response systemically and widespread immunological memory is established (Fahy *et al.*, 1980).

2B 2 Lymphocyte Molecules in Sheep Identified by Specific Monoclonal Antibodies

Monoclonal antibodies have revolutionised the techniques of examining the immune system making it possible to identify and isolate individual components or molecules from a complex mixture of antigens and to study their properties and functions. Until recently, extensive panels of mAbs existed only for the leucocyte molecules of humans and rodents, but a number of groups have now produced mAbs which define the leucocyte molecules of sheep. The review below summarises aspects of the knowledge of the major leucocyte subsets of sheep defined by these mAbs.

CD5 (SBU-T1)

The CD5 molecule (formerly referred to as T1 or Leu-1 in man, MRC OX-19 in rat and Ly-1 in mouse) consists of a single polypeptide chain of 67 kD in all three species, as well as in sheep (Mackay *et al.*, 1985; Beya and Miyasaka, 1986). The tissue distribution of sheep CD5 is similar to that reported for other species. The molecule is displayed on all T cells and thymocytes, and higher levels are expressed on medullary than on most cortical thymocytes (Mackay *et al.*, 1985).

A percentage of normal B cells in human and mouse has been shown to

express the CD5 molecule. In mouse, these $CD5^+B$ cells have long-term survival *in vitro*, secrete IgM autoantibody, have high IgM/low IgG surface expression and are present in high numbers in foetal spleen (Hardy and Hayakawa, 1986). In both man and mouse, the level of expression of CD5 on $CD5^+B$ cells is 5-10-fold less than on T cells (Godal and Ault, 1986). In sheep $CD5^+B$ lymphocytes are most numerous in adult spleen in which approximately 20 percent of B cells weakly express the CD5 molecule. On the other hand, $CD5^+B$ cells in sheep are not detectable within the lymph nodes or efferent lymph, indicating that these cells may be non-recirculating (Mackay, 1988).

The precise function of CD5 has not been defined but CD5-specific mAbs augment the proliferation of T cells *in vitro* in response to various mitogens or alloantigens (Dallman, Thomas and Green, 1984) and, Mackay (1988) claimed to have observed similar results for sheep CD5. Although the ligand for CD5 has not been determined, sequence analysis of human CD5 gene reveals structural features similar to receptor molecules such as the insulin receptor, IL-2R and TfR (Jones *et al.*, 1986). Since mammalian leucocyte molecules are highly conserved through evolution with respect to structure, function and, in many cases, tissue distribution (Ledbetter *et al.*, 1981; Dallman *et al.*, 1984; Mackay and Mackay, 1989) it is possible that some of these receptor molecules may also occur in the sheep CD5 gene structure. CD5 (SBU-T1) has been used as a 'pan T cell' marker in sheep and until CD3 (T3)-specific mAb is produced, CD5 (SBU-T1) will probably remain the best pan T cell marker for sheep (Mackay *et al.*, 1987). CD5 in sheep is identified by the mAb 25-91 (Mackay *et al.*, 1985).

CD4 (SBU-T4)

In man and mouse, the CD4 molecule is a 55-56 kD single chain polypeptide having a characteristic cellular distribution, being expressed by the majority of $CD8^-$ T cells, cortical thymocytes, and cells of the macrophage and langerhans cell lineage(s) (Mackay, 1988). It marks a subset of T cells which recognise antigens in association with MHC Class II molecules and which are usually responsible for helper/inducer activity (Reinherz and Schlossman, 1980; Mason *et al.*, 1983). However, several studies have shown that the $CD4^+$ T cell subset possesses a functional heterogeneity and can, on that basis and on the basis of lymphokine secretion patterns, be subdivided into T_H1 and T_H2 subsets (Janeway, 1975; Marrack and Kappler, 1975; Tada *et al.*, 1978; Swierkosz *et al.*, 1979; Imperiale *et al.*, 1982; Kim *et al.*, 1985; Tite *et al.*, 1985; Mossman *et al.*, 1986; Cherwinski *et al.*, 1987; Francien *et al.*, 1988). The T_H1 subset provides in some cases helper activity for polyclonal B cell differentiation, has cytotoxic

capacity, suppresses some antibody responses, mediates macrophage activation and, produces IL-2, IFN-γ and lymphotoxin (LT). The T_H2 subset is a potent B cell activator, gives antigen-specific help and is marked by the production of IL-4, IL-5, IL-6 and IL-10, (Kim *et al.*, 1985; Tite *et al.*, 1985; Mossman *et al.*, 1986; Killar *et al.*, 1987; Boom, Liano and Abbas, 1988; Coffman *et al.*, 1988).

The CD4 molecule in sheep is similar to those described in human, mouse and rat in its molecular nature, function and distribution (Maddox *et al.*, 1985a; Baldwin *et al.*, 1986; Lalonde *et al.*, 1986). It comprises 70-80 percent of thymocytes and a subpopulation of peripheral T cells (60 percent) whose *in vitro* functional properties are similar to those ascribed to $CD4^+$ cells of man and rodents (Baldwin *et al.*, 1986; Lalonde *et al.*, 1986). In sheep, the CD4 molecule is identified by two mAbs, 44.38 and 44.97, which do not crossreact with other species (Maddox *et al.*, 1985a; Mackay *et al.*, 1986).

CD8 (SBU-T8)

The molecular nature and tissue distribution of sheep CD8 resembles that of other species (Maddox *et al.*, 1985a; Ezaki *et al.*, 1987). It exists as a 36 kD molecule on peripheral lymphocytes but when isolated from thymocytes exists in two polypeptide forms of 33 and 36 kD respectively (Maddox *et al.*, 1985a). CD8 is present on 6-14 percent of peripheral blood lymphocytes, 12-23 percent of efferent lymphocyte, on most cortical thymocytes and on $CD4^-$ medullary thymocytes (Maddox *et al.*, 1985a). In human and rodent, CD8 marks T cells which recognise antigen in association with MHC Class I molecule (Swain, 1983) and which usually exhibit cytotoxic (or suppressor function) and produce large amounts of IFN-γ (Scott and Kaufmann, 1991). The function of sheep $CD8^+$ cells *in vitro* resembles that described for $CD8^+$ cells in other species in that anti-CD8 mAbs are able to block cytotoxic T lymphocyte function (Ezaki *et al.*, 1987). In sheep, CD8 is recognised by three mAbs, 24.96 and 38.65 (Maddox *et al.*, 1985a; Mackay *et al.*, 1986) and, S-T8 (Ezaki *et al.*, 1985; 1987).

γδ-T cell (SBU-T19)

In man and mouse most antigen-specific peripheral T lymphocytes express the T cell differentiation antigens CD4 and CD8, and a T cell receptor (TcR) composed of a CD3-associated disulfide-linked heterodimer, TcR-α/β (Allison *et al.*, 1984; Samelson *et al.*, 1985). A minor population of $CD4^-CD8^-CD3^+$ (double negative, DN) cells has also been identified in the thymus and peripheral organs of these species (Reinherz and Schlossman, 1980; Fowlkes *et al.*, 1985; Pardoll *et al.*, 1987). Immunoprecipitation studies of murine DN T cells have shown that they do

not express cell surface TcR-α/β; rather they express the TcR-γ/δ complex (Nakanishi *et al.*, 1987; Pardoll *et al.*, 1987; Cron *et al.*, 1988).

DN T cells are also present in the sheep and recently an antigen designated T19, of high molecular weight (220 kD), has been identified on the surface of most of the peripheral DN T cells (Mackay *et al.*, 1986; Miyasaka *et al.*, 1988). This antigen is recognised by mAbs 19-19 (Mackay *et al.*, 1986) and 197 (McClure *et al.*, 1989). T19 defines a lymphocyte subset which is $CD5^+CD4^-CD8^-$ but sIg^-. T 19^+ cells in the peripheral lymphoid organs are found almost exclusively in the small lymphocyte population, exhibit the physical characteristics of normal recirculating lymphocytes, and are responsive to T cell mitogens and not to B cell mitogens (Mackay *et al.*, 1986). The tissue distribution of $T19^+$ cells reveals a predominance in peripheral blood and afferent lymph where, depending on the age of the animal, they may represent 6-30 percent of lymphocytes, with reduced numbers in lymph node, efferent lymph and thymus (Mackay *et al.*, 1986; Maddox *et al.*, 1987a; 1987b; 1987c; Mackay, 1988; McClure *et al.*, 1989).

Although there is no specific mAb for CD3 of sheep, it has been characterised using rabbit anti-human CD3 antiserum which also reacts with the sheep CD3 molecule (Hein, W., unpublished, 1988). Using this antiserum it has also been shown that in sheep, $T19^+$ cells express the CD3 molecule (Hein and Mackay, 1991). Since in human and mouse, $CD3^+CD4^-CD8^-$ cells express the γ and the δ chains of the TcR, it is likely by analogy that the $CD3^+CD4^-CD8^-T19^+$ lymphocytes in sheep also express the γ and δ chains of the TcR. In fact, Northern blot hybridisation using sheep α, β, γ and δ probes showed that $T19^+$ (197^+) cells express γ and δ message (Hein *et al.*, 1989; Mackay, Beya and Matzinger, 1989; Clevers *et al.*, 1990).

In human and mouse, functional studies of γδ T cells isolated from peripheral T cell populations and activated with antigen-independent stimuli such as mitogens or mAbs, have shown that they develop non-MHC-restricted cytolytic activity (Brenner *et al.*, 1987; Borst *et al.*, 1987). Thus, it has been proposed that γδ T cells in epithelial regions function in disease surveillance and could eliminate in a cytotoxic fashion, transformed epithelial cells and those infected by pathogens from the environment (Janeway *et al.*, 1988; Goodman and Lefrancois, 1988; Brandtzaeg *et al.*, 1989). Furthermore, the abundance of γδ T cells in epithelia of skin may provide a unique role for them in the recognition of certain infectious agents presented in the context of modified Class I antigens, since it was demonstrated that mouse alloreactive γδ T cell lines and clones were both cytolytic and released lymphokines after recognition of a non-classical H-2 antigen encoded in the TL region of the MHC (Bluestone *et al.*, 1988). In addition, γδ TcR^+ intestinal intra-

epithelial lymphocytes (IEL) spontaneously produced IFN-γ and IL-5 and possessed large quantities of cytokine-specific mRNA, an indication that they were programmed for cytokine production (Taguchi *et al.*, 1991).

Results from functional studies of ruminant γδ T cells are limited and inconclusive. Sheep γδ T cells generally show poor proliferative responses when stimulated *in vitro* with T cell mitogens but, in allogeneic mixed lymphocyte cultures, they can proliferate vigorously and may even overgrow the α/β population (McClure and Hein, 1989; Hein and Mackay, 1991). In some assays, sheep γδ T cells exhibit cytotoxic activity against allogeneic cells, although the MHC restriction pattern of the response was inconsistent (Mackay *et al.*, 1988b). It has been speculated that in sheep, γδ T cells may have an immunoregulatory function in peripheral immune responses to antigen, exercised via their contribution to the various effector molecules. This derives support from the fact that *in vitro* mitogenic stimulation of T19 enriched cells resulted in a substantial decrease in the expression of the antigen and was accompanied by the appearance of $CD4^+$ and/or $CD8^+$ cells (McClure and Hein, 1989). It is too early to assess the role of γδ T cells in ruminants during the pathogenesis of disease. However, γδ T cells infiltrate lesions in sheep skin caused by infection with orf virus (Hein and Mackay, 1991). In addition, significant numbers of γδ T cells have also been observed within liver leucocyte foci in sheep challenged with *Taenia hydatigena* and interestingly, appreciable numbers of the cells were also present in well developed germinal centres in the draining hepatic lymphnode (Meeusen *et al.*, 1990). These observations clearly suggest that in some circumstances γδ T cells are selectively recruited to sites of active inflammation.

CD45 (SBU-LCA) Leucocyte Common Antigen

The CD45 molecule of mammals (formerly LCA or T200) is expressed by all leucocytes although different cell lineages or cells at various stages of differentiation express different forms of the molecule (Mackay, 1988). In sheep, the mAb 1-32 defines an antigen which, based on tissue distribution and MW, is an analogue of the CD45 or LCA of human, mouse and rat. In sheep, the antigen is present only on cells of the haemopoietic lineage and is expressed on the surface of all lymphocytes of the thymus, spleen, lymph nodes, ileal Peyer's patches (IPP) and peripheral blood, as well as being found on macrophages and granulocytes (Maddox *et al.*, 1985b). SDS-PAGE analysis of LCA isolated from lymphocytes reveals molecular forms of 190 kD, 210 kD and 225 kD. However, different molecular forms of ovine LCA were found on different lymphocyte populations, with B cells lacking the 210 kD form found on T cells and thymocytes, and thymocytes lacking the 225 kD form found on B cells. One interesting aspect of ovine CD45 is the presence

of the 190 kD molecule on B cells from IPP which contrasts with the 225 kD form on peripheral B cell CD45 (Mackay *et al.*, 1987). This finding most probably relates to the unique nature of IPP as a primary B cell lymphoid organ (Reynolds and Morris, 1983) and indicates that the majority of B cells in IPP are 'immature'.

Since only one CD45 gene has been identified, the various molecular species of LCA probably result from alternative splicing of mRNA (Saga *et al.*, 1986; Thomas and Lefrancois, 1988) and in fact, mAbs have been produced to the regions of CD45 with restricted expression. Thus, the mAb B220 in the mouse reacts mostly with B cells (Coffman and Weissman, 1981) and the epitope for this antibody is probably located on that portion of CD45 expressed only by B cells. A similar mAb, 20-96 has been produced against the P220 component of sheep CD45 displaying a restricted tissue distribution (Mackay *et al.*, 1987). This antibody reacts with all B cells and a subpopulation of $CD4^-$,$CD8^-$,$T19^-$ and sIg^- lymphocytes. The function of CD45 is not clear but it is thought to be essential for the activation of protein tyrosine kinase during lymphocyte triggering. It has been claimed to participate in lysis by cytotoxic T cells and NK cells (Newman *et al.*, 1983) as well as in B cell differentiation (Yakura *et al.*, 1983).

Major Histocompatibility (MHC) antigens (Ovine Leucocyte Antigens, OLA)

The MHC complex is a chromosomal region that contains loci coding for molecules that are involved in self recognition. There are two major loci namely, Class I and Class II. The Class I gene products or MHC Class I antigens (H-2 K,-D, and -L in mouse; HLA-A, -B, and -C in human) are found on every nucleated cell in varying amounts and function as targets for cytotoxic T lymphocytes. They are classical alloantigens consisting of a polymorphic glycosylated polypeptide chain of 40-45 kD (heavy chain) in close noncovalent association with an oligomorphic non-glycosylated light chain (β-2 microglobulin) of approximately 13 kD (Male, Champion and Cooke, 1987). The Class II gene products or MHC Class II antigens; I-A, I-E in mouse; HLA-DR,-DC and -SB in human (Klein, 1978; Schwartz and Cullen, 1978; Shackelford *et al.*, 1982; Kaufman *et al.*, 1984) are normally found as heterodimeric cell surface glycoproteins consisting of a heavy (α) chain of 33-38 kD and a light (β) chain of 25-28 kD (Ferrone, Allison and Pellegrino, 1978; Schwartz and Cullen, 1978) and are expressed mainly on B cells, activated T cells and on cells of the monocyte/macrophage lineage (Hammerling *et al.*, 1975; David *et al.*, 1976). The MHC Class II molecules are recognised by the helper/inducer T lymphocyte subpopulations.

In sheep, both the Class I and Class II MHC antigens have been characterised using mAbs. Three mAbs, SBU-I 41-17; SBU-I 41-19 and SBU-I 41-

28 have been generated against MHC Class I antigens which identify all peripheral lymphocytes, but only 20-40 percent of thymocytes (Gogolin-Ewens *et al.*, 1985). They all precipitate a 44 kD heavy chain that is noncovalently linked with a 12 kD light chain. Similarly, mAbs SBU-II 28-1, 38-27 and 38-64 (Puri *et al.*, 1985) and SW73.2 (Hopkins *et al.*, 1986) believed to react with sheep MHC Class II antigens have been produced. The antigens immunoprecipitated with these mAbs all gave typical two-chain patterns of 30-32 kD/24-26 kD (Puri *et al.*, 1985) and 33-35/28-30 kD (Hopkins *et al.*, 1986) by SDS-PAGE and could be detected on majority of B cells, a small proportion of sIg^- cells in the blood, macrophages in afferent lymph and reticular epithelial cells in the thymic cortex and medulla. The MHC Class II molecules have proven to be useful markers for assessing the state of activation of T cells in different lymphoid compartments and after antigenic stimulation and, are also involved in antigenic recognition by $CD4^+$ T cells (Mackay *et al.*, 1986; 1987).

CD1 (SBU-T6)

This thymic antigen, termed SBU-T6 in sheep, is similar to CD1 or T6 in man and has been characterised in sheep by Mackay *et al.* (1985) using the mAb 20-27. Its distribution in sheep differs slightly from that of T6 in human since SBU-T6 is detectable at low levels on peripheral blood B cells. However, its pattern of expression on Langerhans cells and cortical thymocytes is conserved in all species. SBU-T6 is a 46 kD polypeptide which associates with a 12 kD subunit presumably representing sheep β-2 microglobulin. In addition to staining cortical thymocytes and Langerhans cells, monoclonal anti-SBU-T6 also reacts with dendritic (veiled) cells in afferent lymph and with dendritic cells within the paracortical areas of the lymph node (Mackay *et al.*, 1987).

B Cell Antigens

B cells recognise antigens by their endogenously synthesised surface immunoglobulin, which is the characteristic B cell marker. During differentiation, B cells initially acquire sIg of the IgM class, which acts as their antigen receptor. In subsequent development they may change the class of their sIg, but their antigen specificity remains the same. Surface Ig is present on all B cells but is not expressed when they terminally differentiate into plasma cells. Detection of sIg in sheep has depended on the use of heteroantisera (Beh *et al.*, 1979; Husband *et al.*, 1979; Gerber *et al.*, 1985). However, mAbs have been produced which discriminate B cells from T cells in sheep (Miyasaka *et al.*, 1985). The mAb VPM 30 is an IgM monoclonal specific for sheep B cells (J Hopkins, personal communication).

CHAPTER THREE

General Materials and Methods

3.1 Experimental Animals

Finnish Landrace (Finns) or Suffolk breeds of sheep aged between six to 18 months were purchased from Moredun Research Institute, Edinburgh. Those used for peripheral blood studies were housed in an air-conditioned, fly-proof urban isolation unit whereas sheep used for efferent lymphatic cannulation studies were kept in metabolism cages in the isolation unit at the Centre for Tropical Veterinary Medicine, Edinburgh. All the animals were dewormed with 5 mg kg^{-1} dose of Fenbendazole (Panacur™; Hoechst, Frankfurt, Germany) before use in experiments. They were fed *ad libitum* on hay and water with regulated supply of concentrates.

3.2 Trypanosomes

T. evansi TREU 2143 was used throughout the course of study. It was a cloned derivative of *T. evansi* TREU 1558, originally isolated from a naturally infected camel in Aroma, Kassala Province of Sudan in 1977 as *T. evansi*/KASSALA/77/KAS/4. Appendix I details the cloning of TREU 2143.

3.2.1 Subpassage in mice

T. evansi TREU 2143 was cryopreserved in liquid nitrogen in sealed capillary tubes. For use, two capillary tubes were rapidly thawed and their contents suspended in 1 ml sterile phosphate buffered saline glucose (PSG), pH 8.0 (Appendix II(1). Two mice treated three days previously with 300 mg kg^{-1} of cyclophosphamide (CPA) were inoculated intraperitoneally with 200 µls of trypanosome suspension. Three days later, 400 µls of blood were expressed from the tail vein of the mice and further passaged in two CPA-treated mice.

3.2.2 Separation of *T. evansi* from Mouse Blood

The second set of infected mice were exsanguinated by sterile open cardiac puncture under terminal anaesthesia using diethylether, 6-7 days p.i. when they had developed fulminating parasitaemia. Trypanosomes were then separated from the blood by anion exchange chromatography on a column of sterile diethylaminoethyl cellulose, DEAE-52 (Whatman Chemical Separation Ltd, Kent, England) as described by Lanham and Godfrey (1970). This technique is detailed in Appendix II(2) and III(1).

3.2.3 Establishment of Infection in Sheep

The neck of each animal was clipped and sterilised by scrubbing with cotton wool soaked in 70 percent alcohol. Sheep were infected by inoculation into the external jugular vein of 2×10^6 *T. evansi* TREU 2143 in 200 µls of sterile PSG.

3.3 Clinical and Parasitological Parameters

3.3.1 Body Temperature

The body temperature of sheep used in the peripheral blood experiments was monitored daily using a digital electronic thermometer.

3.3.2 Packed Cell Volume (PCV)

Routine haematology and assessment of parasitaemia were performed on 2 ml blood taken by jugular venipuncture into 5 ml EDTA coated plastic vials. Blood for PCV was drawn into microcapillary tubes up to two-thirds of their capacity, which were then heat-sealed at one end. The capillaries were then centrifuged at 12,000 rpm in a microhaematocrit centrifuge (Biofuge, Heraeus Sepatech) and the PCV determined using a microheamatocrit reader (Hawksley).

3.3.3 Detection of Trypanosomes in Blood and Lymph

Blood parasitaemia was monitored by the examination of unstained wet blood films on a clean slide covered with 22 x 22 mm^2 coverslip. A systematic examination was made using a phase contrast microscope at x400 magnification for 200 fields. Parasitaemia was represented as numbers of trypanosomes per 200 fields. Where no parasites were detected by this technique, the haematocrit centrifugation technique (HCT) for parasite detection as described by Woo (1970) was done and examined under x100 magnification.

In lymph, trypanosomes were concentrated by centrifugation at 2260 r.p.m. for 20 minutes at $4^{o}C$ and detected by examining the resuspended pellet.

3.3.4 Total and Differential Leucocyte Counts

Total leucocyte count (TWBC) of blood was determined using a Coulter cell counter (Coulter Electronics Ltd, Harpenden, U.K.). Differential counts were made using Giemsa stained thin blood smears and were expressed as percentages of 1000 leucocytes counted. Lymph cell counts were made in a System 9000 automated cell counter (Serono, Baker Diagnostics, Allentown, Pennsylvania, U.S.A.) which also discriminated small lymphocytes from blast cells. Differential cell counts were determed using Giemsa stained smears.

3.4 Separation of Peripheral Blood Leucocytes

Peripheral blood leucocytes were separated either by density gradient centrifugation of defibrinated blood over lymphoprep (Boyum, 1968; Ling and MacLennan, 1981) or by hypotonic lysis of erythrocytes using tris-ammonium

chloride (Mishell and Shigi, 1980). Density gradient centrifugation yields a relatively pure peripheral blood mononuclear cells since it separates them from erythrocytes, granulocytes and platelets. Separation by hypotonic lysis on the other hand is simpler and quicker but yields a mixed population of all other WBC elements. These different populations can, however, be analysed separately in flow cytometry by 'live gating' using the different physical parameters of the cells. Detailed protocols of the density gradient centrifugation and hypotonic tris-ammonium chloride lysing techniques used are given in Appendices III(2) and III(3) respectively.

3.4.1 Preparation of Cytospin Smears

Cytosmears were made using a cytocentrifuge (Shandon Southern Instruments Ltd, Cheshire, U.K.). 80 µls of cell suspension at 10^5 cell ml^{-1} was centrifuged at 800 r.p.m. for 5 minutes. Smears were air dried, fixed for 2 minutes in technical methanol (95 percent, BDH) and stained in 10 percent Giemsa.

3.5 Phenotypic Analysis of Peripheral Blood Leucocytes and Efferent Lymphocytes

3.5.1 Monoclonal Antibodies and Immunoconjugates

A panel of 11 mAbs were used to analyse the dynamics of various leucocyte phenotypes in sheep. Nine of the mAbs were obtained from the Sheep Biology Unit (SBU), School of Veterinary Science, University of Melbourne, Australia, as lyophilised ascitic fluid, reconstituted in 5 mls of sterile distilled water and stored in 200 µl aliquots at -20°C until used. Two mAbs were provided by Dr J Hopkins of the Department of Veterinary Pathology, University of Edinburgh, as tissue culture supernatants and were also stored at -20°C in 1 ml aliquots until used. In cases of mAbs which identify antigens analogous to those described in other species such as human and mouse, the cluster of differentiation (CD) nomenclature (Bernard *et al.*, 1984) has been adopted and applied to them. Table 3.1a lists the mAbs used and some of their biological characteristics.

The immunoconjugates used are listed in Table 3.1b. For single colour flow cytometry, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (GAM/Ig/FITC) and goat anti-rabbit immunoglobulin (GAR/Ig/FITC) [Nordic immunochemicals] were used as second antibodies. For two colour flow cytometry, biotinylated anti-cell (SBU) antibodies, including biotinylated VPM30 (anti-sheep B cell), for red fluorescence, and non-biotinylated anti-sheep mAbs for green fluorescence were used as first antibodies. Red fluorescence was visualised with R-phycoerthrin-streptavidin (PE-SA) complex (Amersham, U.K.) while

Table 3.1a **Characteristics of anti-sheep monoclonal antibodies.**

Monoclonal antibodies	Isotype	Antigen specificity	Cells identified
21-91 (SBU-T1)	IgG_1	CD5	T cells and a subpopulation of B cells
44-38 (SBU-T4)	IgG_{2a}	CD4	MHC Class II restricted T cells
38-65 (SBU-T8) S-T8	IgG_{2a} IgM	CD8	MHC Class I restricted T cells
19-19 (SBU-T19)	IgG_1	T-19	$\gamma\delta$ T cells
20-27 (SBU-T6)	IgG_1	CD1	Cortical thymocytes, dendritic cells and some B cells in peripheral blood
28-1 (SBU-II)	IgG_1	MHC Class II	B cells, activated T cells, some macrophages, epithelia and dendritic cells
20-96 (SBU-LCA p220)	IgG_1	CD45R	B cells and some T cells
VPM 30	IgM	B cell antigen	All B cells
41-19 (SBU-I)	IgG_1	MHC Class I	All somatic cells
1-28 (SBU-LCA)	IgG_2a	CD45	All leucocytes
F(ab') RAS 1g	Polyclonal	sIg	B cells
<u>For microELISA</u>			
McM1	IgG_{2a},κ		IgG_1(pFc')
McM3	IgG_3,κ		IgG_2
McM6	IgG_3,κ		L-chain
McM9	IgG_3,κ		IgM
McM10	IgG_2a,κ		IgA

Table 3.1b **Immunoconjugates**

Antibody	Dye	Specificity
1. <u>For Immunofluorescence</u>		
Sheep anti-mouse Ig	Fluorescein isothiocyanate (FITC)	anti-IgG_1
Sheep anti-mouse Ig	Fluorescein isothiocyanate (FITC)	anti-IgG_{2a}
Sheep anti-mouse Ig	Fluorescein isothiocyanate (FITC)	anti-IgM(Fc)
Goat anti-mouse Ig	Fluorescein isothiocyanate (FITC)	anti-Ig
Goat anti-rabbit Ig	Fluorescein isothiocyanate (FITC)	polyvalent
2. <u>For microELISA</u>		
Donkey anti-sheep/ Goat Ig	Horseradish peroxidase (HRP)	anti-IgG
Polyvalent Goat anti-mouse Ig	HRP	anti-IgA, -IgG, and -IgM

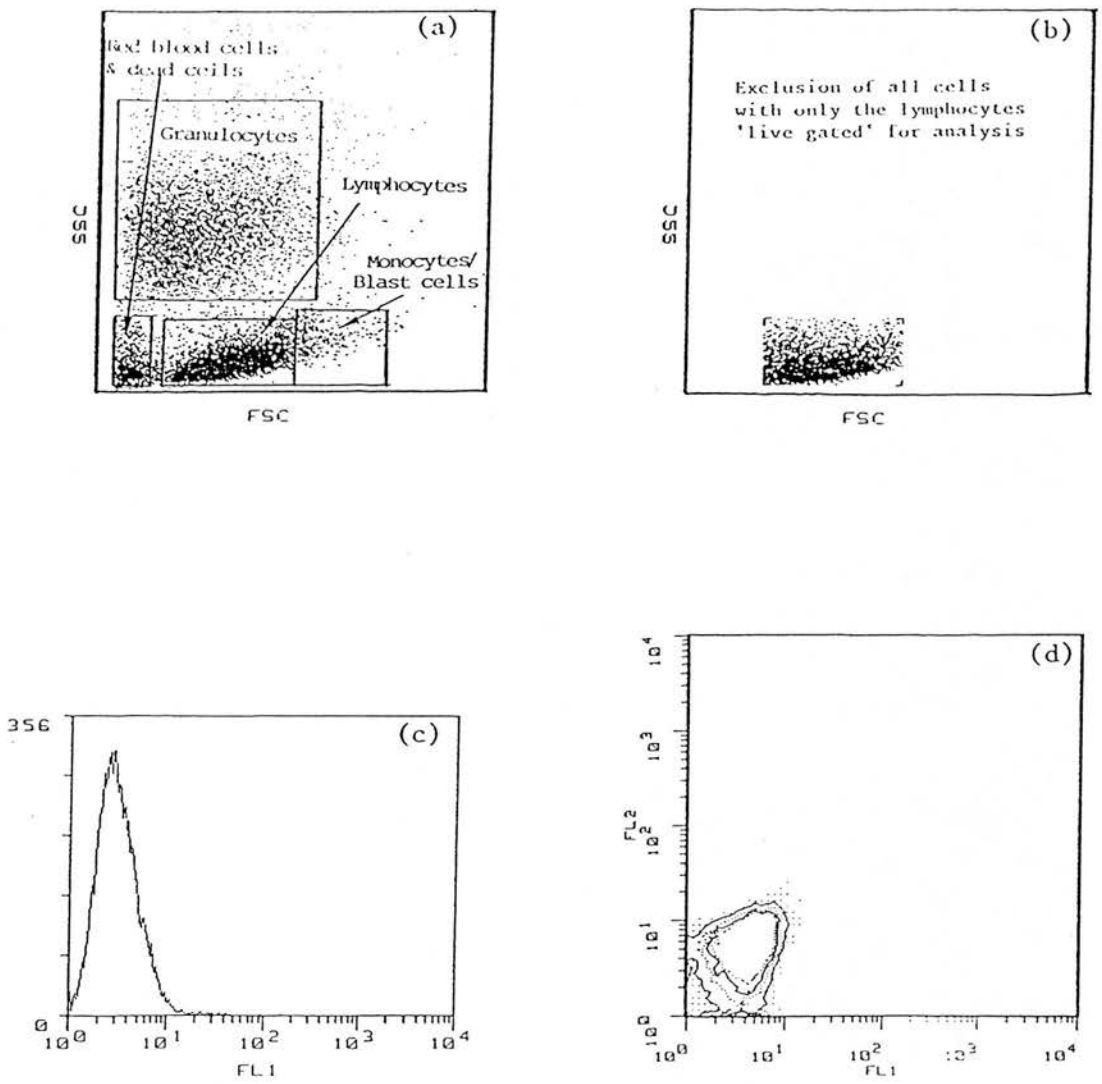


FIGURE 3.1 Flow cytometry display of PBLs for 'live gating' of lymphocytes using forward and side angle light scatter (FSC and SSC).

(a) Dot plot display (ungated) of a negative control sample showing the distribution of the various blood elements based on their physical characteristics.

(b) Exclusion of dead cells, rbcs, granulocytes and monocytes by 'live gating' the lymphocytes.

(c) Frequency histogram display of the 'live gated' lymphocytes.

(d) Contour profile of the negative control sample of a 2-colour staining adjusted for optimal FITC and RPE voltage levels.

green fluorescence was visualised with isotype specific FITC-sheep anti-mouse Ig (ShAM/Ig/FITC) conjugates (The Binding Site,U.K.)

3.5.2 Flow Cytofluorimetry and Analysis of Cytometric Data

Flow cytometry is an analytical technique which in a population of continuously flowing fluorochrome labelled cells permits accurate quantitation of various parameters of individual cells, such as the distribution of fluorescence intensities and their sizes. Data on these parameters can be stored and analysed in a computer. In this work, flow cytometric acquisition of fluorochrome labelled cell data was carried out using a fluorescence-activated cell analyser, FACSCAN (Becton Dickinson, Mountain View, CA, U.S.A.). The Consort 30 programme was used in the acquisition, storage and analysis of both the single and two colour flow cytometric parameters of the labelled sheep peripheral blood and efferent lymph lymphocytes. In each case data was derived by analysing 10,000 cells (or events) per sample.

For single-colour parameters, the cytometer configurations were calibrated and set using negative control cells stained with normal mouse serum (NMS) and the appropriate immunoconjugates. FITC fluorescence (520 nM) emission was detected with the photomultiplier tube voltage set at 555 mV. Forward (FSC) and side or 90 degree (SSC) scatter amplifications were linear and set at E00 and 326 respectively, while FITC amplification was logarithmic (all 256 channels). Using dot plot (Figure 3.1a), lymphocytes were 'live-gated' for acquisition and analysis while cell debris, dead cells, red blood cells (RBCs), granulocytes and monocytes were excluded on the basis of forward and side angle light scatter (Figure 3.1b). Forward-angle light scatter, 90-degree light scatter and fluorescent data were plotted as frequency histograms with the log fluorescent intensity presented on the x-axis and the frequency of cell numbers presented on the y-axis (Figure 3.1c).

For two-colour parameters, the cytometer configurations were calibrated and set using the negative control cells stained with appropriate concentrations of NMS-biotin and negative mAb, and then visualised with PE-SA and the FITC conjugated anti-immunoglobulin. The FSC and SSC configurations were the same as above. By using dot plot of FSC vs SSC, the lymphocyte cluster was 'live-gated' while the unwanted clusters were excluded (Figure 3.1b). Using the autofluorescence (in the FL1 vs FL2 parameter option) of the unstained negative control lymphocytes inside the monitor gate, the optimum FITC and RPE voltage levels were adjusted to the lower left corner of the box (Figure 3.1d). These optimal PMT voltages were found to be 625 mV and 574 mV for FITC and RPE respectively, and both FL1 (FITC) and FL2 (RPE) were in the logarithmic amplification. To compensate for the unwanted spectral overlap of emitted light from the FITC and

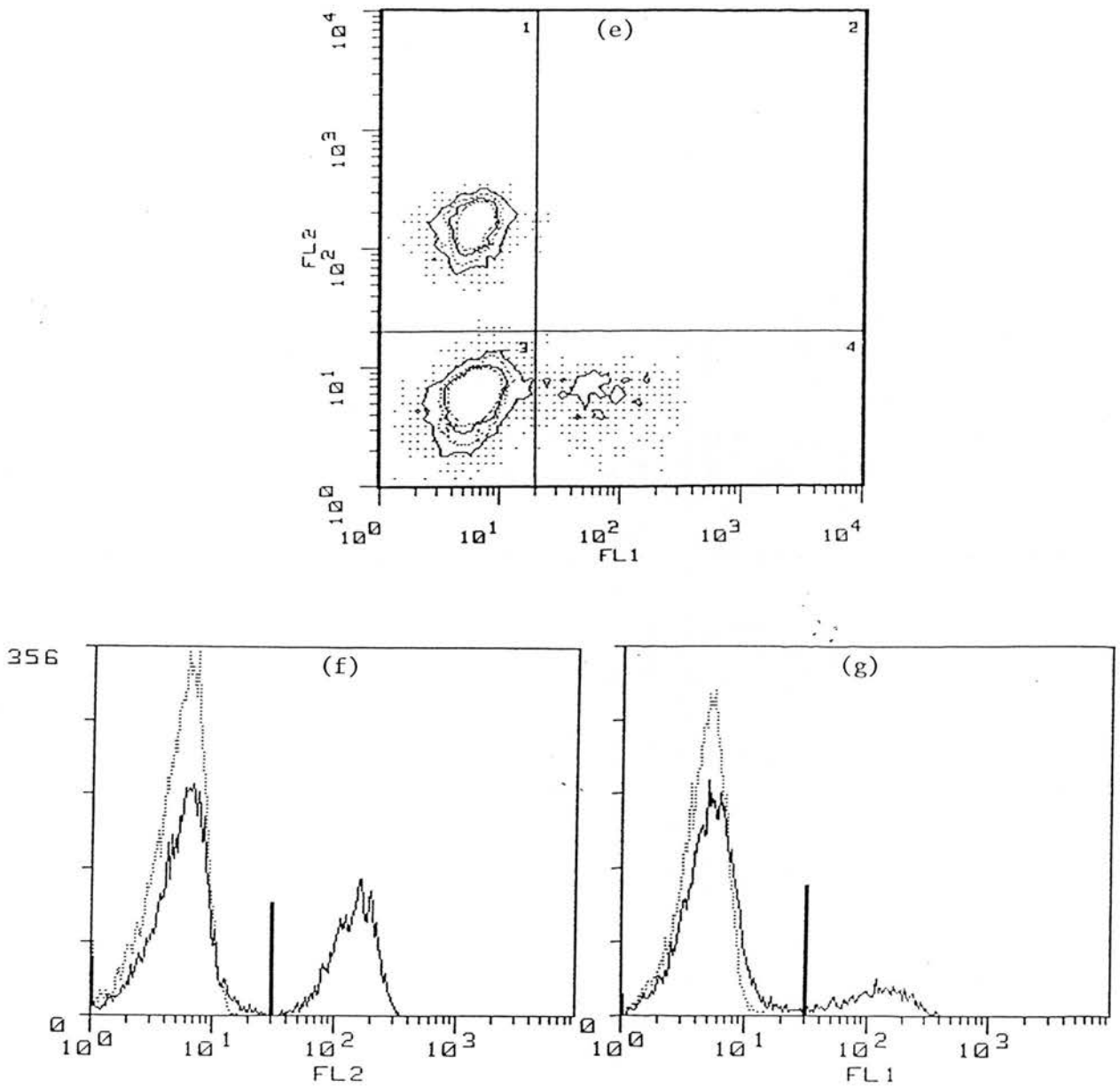


FIGURE 3.1 (e) Two-colour immunofluorescence contour display of PBLs stained for $CD4^+$ (FL2) and $CD8^+$ (FL1) cells. Quadrant 1 = $CD4^+$ cells; 2 = $CD4^+CD8^+$ cells (none in this case); 3 = negative cells, i.e. $CD4^-CD8^-$; 4 = $CD8^+$ cells.

(f) Frequency histogram of the $CD4^+$ cells: (.....) = negative control sample; (--------) = stained cells; The first peak represents the $CD4^-$ cells while the second peak (right of marker) is the $CD4^+$ cells.

(g) Frequency histogram of the $CD8^+$ cells: (.....) = negative control; (--------) = stained cells: First peak = $CD8^-$; second peak = $CD8^+$ cells.

RPE dyes used, the FL1-% FL2 PMT voltage was set at 9 mV to remove PE signal from the FL1 region, while the FL2-% FL1 PMT voltage was set at 30 mV to remove FITC signal from the FL2 region. All parameters for two-colour immunofluorescence were acquired in the LIST DATA structure which allows for the presentation of data in frequency histograms as in uncorrelated single-colour parameter, as well as allowing the generation of various combinations of dual parameter correlations such as FSC vs SSC dot plot and FL1 vs FL2 contour plot. Contour plot (graph) maps events in a 64 x 64 channel area defined by the selected parameters, with contour lines representing the cell density distribution over this area. The result corresponds to a geographical topographic map, which distinguishes between altitude differences of a terrain, i.e. mountains (increasing cell densities or peaks) and valleys (areas of low cell densities). For statistical data analysis, the contour graphs in this study were set into calculation regions defined by setting quadrant markers or windows. Figure 3.1e represents a CD8 FITC/CD4 PE system in which quadrant 1 represents CD4 (PE) positives only, quadrant 2 represents CD4/CD8 (FITC/PE) dual positives (in this experiment there are no dual positives), quadrant 3 represents the negative population for both antibodies while quadrant 4 is the CD8 (FITC) positives only. Figures 3.1f and 3.1g represent frequency histograms generated for $CD4^+$ and $CD8^+$ cells respectively from the sample parameter.

3.5.3 Protocols for Indirect Immunofluorescence Staining

3.5.3.1 Single Colour Flow Cytometry

Slight modifications of the methods of Mackay *et al.* (1988a) and Hudson and Hay (1989) were adopted in this study. Briefly, 5×10^5 ovine peripheral blood or efferent lymphocytes in ice cold immunofluorescence buffer [IFB] (Appendix II(3)) were dispensed in 50 µl aliquots into round-bottomed mini-test tubes (Sterilin). Each was treated with 25 µls of appropriately diluted mAbs, mixed by gentle tapping and incubated on ice (4^oC) for 30 minutes. The cells were then washed twice in 800 µls of IFB by centrifugation, resuspended in 50 µls of IFB and labelled with 25 µls of an optimal dilution of the appropriate antibody conjugate or PE-SA complex. They were incubated again in the dark for 30 minutes at 4^oC, washed twice (for FITC stained cells) or once (for PE stained cells) as before and resuspended in 200 µls of IFB. Cells were usually analysed on the same day, but if this was not possible, the cells were fixed by resuspension in equal volumes (100 µls each) of IFB and freshly made one percent paraformaldehyde fixative (Appendix II(4)), wrapped in tinfoil, stored at 4^oC and analysed the following day. The control samples were treated in exactly the same way except that instead of a mAb they were reacted with NMS or biotinylated NMS (BNMS).

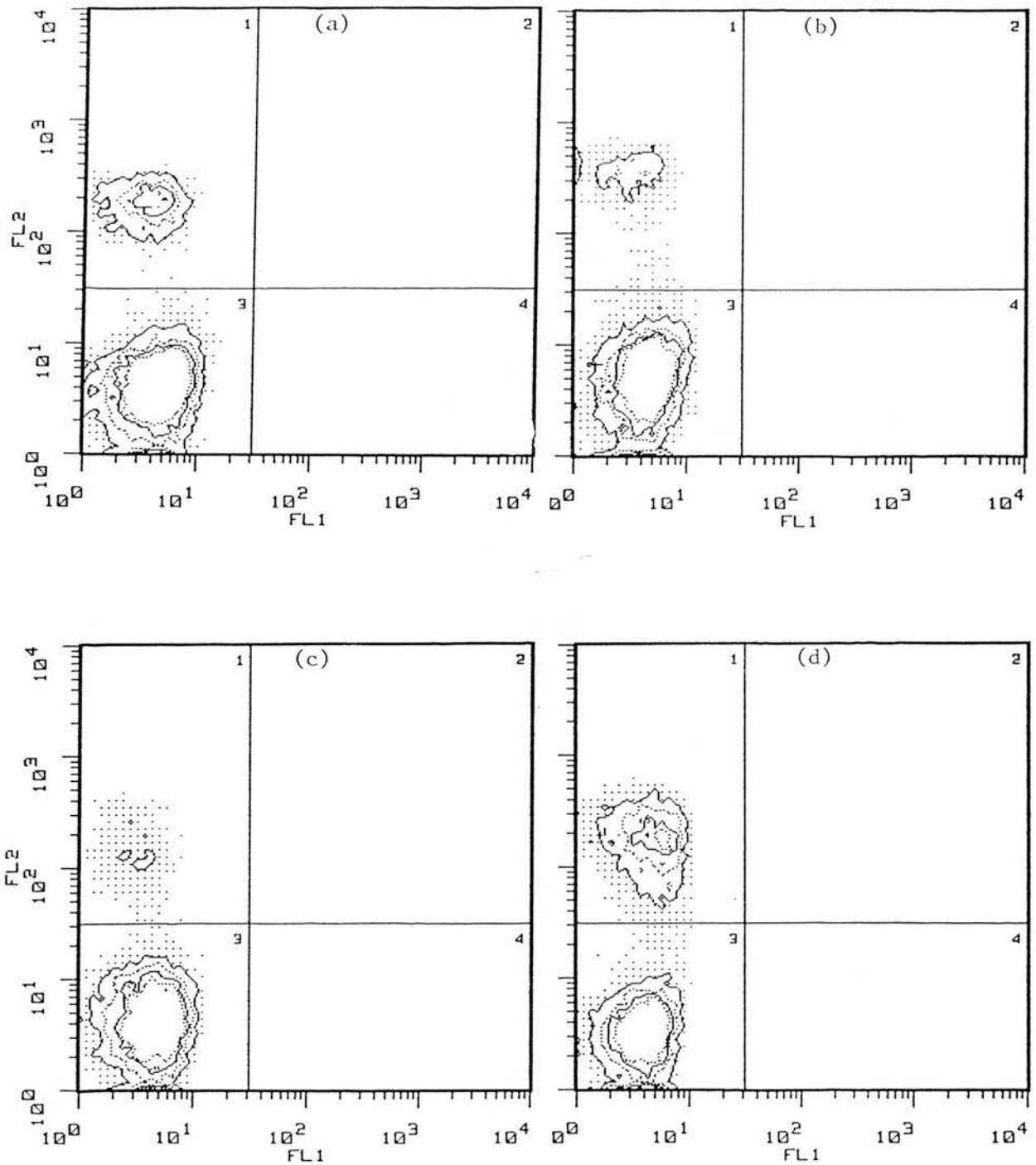


FIGURE 3.2 Contour profiles of cells stained with SBU-T4, SBU-T8, SBU-T19 and VPM 30 after the antibodies were biotinylated.

(a) $CD4^+$ cells (quadrant 1)
(b) $CD8^+$ cells (quadrant 1)
(c) $T19^+$ cells (quadrant 1)
(d) VPM 30^+ cells (quadrant 1)

3.5.3.2 Two-colour Flow Cytometry

For this technique, a modification of the method of Loken, Parks and Herzenberg (1977) was adopted. Cells were dispensed as above and treated simultaneously with 25 µls each of the non-biotinylated and biotinylated mAbs as appropriate. They were incubated as above and washed three times in IFB. 25 µls each of the appropriately diluted PE-SA and isotype specific FITC-conjugated second antibodies were again simultaneously added for visualisation of the red and green fluorescence respectively. They were then incubated at $4^{o}C$ in the dark for 30 minutes, washed once and analysed. Negative controls were stained with BNMS and an anti-metacyclic *T. congolense* mAb as the first antibody.

3.5.4 Biotinylation of Monoclonal Antibodies

SBU-T4, SBU-T8, SBU-T19 and VPM 30 were biotinylated for use in two-colour flow cytometry according to the method of Hudson and Hay (1989). Briefly, Ig isotypes from 1 ml ascitic fluid or 5 mls tissue culture supernate (VPM 30) were precipitated by treatment with ammonium sulphate at 45 percent saturation (v/v). The precipitates were centrifuged at 1000 g for 15 minutes at $4^{o}C$, redissolved in 1 ml of 0.1 M bicarbonate buffer, pH 8.4 (Appendix II(6)) and dialysed extensively in bicarbonate buffer. The protein concentration was determined and adjusted to 1 mg ml^{-1} in buffer. Immunoglobulins were biotinylated by the addition of biotin-amido caproate N-hydroxysuccinimide ester (Sigma) in dimethyl sulphoxide (DMSO) at 2 mg ml^{-1} at a biotin: protein ratio of 75 µg:1 mg. They were mixed together and rotated at room temperature for 4 hours. The biotinylated antibodies were then dialysed against three changes of 0.1 M Tris-Hcl buffer, pH 8.4 (Appendix II(9) for 72 hours at $4^{o}C$. Figure 3.2 illustrates contour profiles of peripheral blood leucocytes stained with the biotinylated antibodies.

3.5.5 Optimisation of mAb and Conjugate Dilutions

All mAbs and conjugates were titrated for optimal working dilutions except for S-T8 (T8 basal) and VPM 30 which were used as undiluted tissue culture supernates. The biotinylated SBU-T4, SBU-T8, SBU-T19 and VPM 30 as well as the $F(ab')_2$RAS serum were titrated at doubling dilutions from 1:25 to 1:400 whereas SBU-T6 and SBU-T19 were titrated from 1:125 to 1:1000. All other mAbs, NMS and the anti-metacyclic *T. congolense* mAb were tested at doubling dilutions from 1:250 to 1:2000. Sheep peripheral blood leucocytes were stained for flow cytometry with these dilutions. Red and green fluorescence were visualised by PE-SA and FITC conjugates at arbitrary dilutions of 1:200 and 1:80 respectively. The optimal working dilution of each of the antibodies chosen was that which gave the strongest

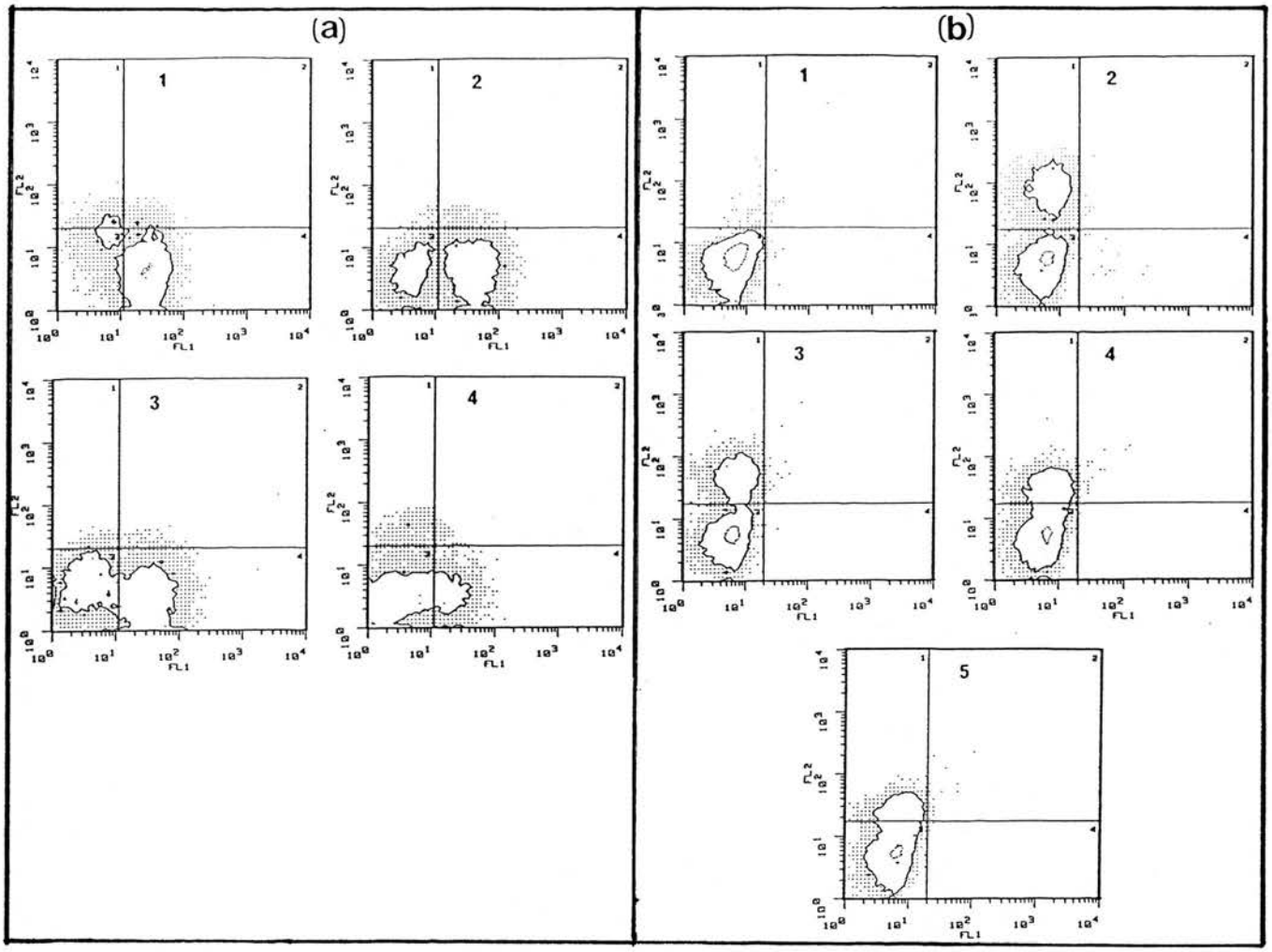


FIGURE 3.3 Optimisation of working dilutions of monoclonal antibodies by titration.

(a) $CD5^+$ cells at various dilutions of SBU-T1 mAb
1. 1:250 dilution
2. 1:500 dilution
3. 1:1000 dilution
4. 1:2000 dilution

(b) B cells at various dilutions of VPM 30 mAb
1. Negative control
2. 1:50 dilution
3. 1:100 dilution
4. 1:200 dilution
5. 1:400 dilution

In each case the dilution which gave the best definition between the negative and positive population was chosen. In this case 1:500 for SBU-T1 and 1:50 for VPM 30.

fluorescence based on the mode and mean channel fluorescence intensities and, a clear distinction (within normal range) between the positive and negative populations.

The optimal working dilutions of the various conjugates and the PE-SA complex were determined by testing different dilutions against the optimal dilutions of the mAbs, and the dilution giving strong specific fluorescence with low background staining of the negative control cells was chosen. Figures 3.3a,b represent FACSCAN frequency histograms and contour profiles of SBU-T1 and biotinylated VPM 30 at various dilutions. The profiles of the rest of the mAbs and conjugates are not shown because they were destroyed in an arson attack at the Centre for Tropical Veterinary Medicine (CTVM) in November 1991. Table 3.2 lists the working dilutions chosen for each of the mAbs and conjugates.

3.5.6 Comparison of Cell Separation Techniques

To assess which cell separation technique would give the best result in the study, separation by density gradient centrifugation and hypotonic lysis were compared. Leucocytes isolated by each method were stained for CD5, CD4, CD8, γδ T cell and B cells using appropriate mAbs and then analysed by flow cytometry. The analysis revealed that a significant proportion of lymphocyte subsets was lost when separated by density gradient centrifugation over lymphoprep in comparison to those separated by hypotonic red blood cell lysis using tris-NH_4Cl (Figure 3.4). As a result, hypotonic lysis was adopted and used in all cell separation for immunocytofluorimetric studies.

3.6 Surgical Cannulation of the Efferent Lymphatic Duct of the Prefemoral Lymph Node

The prefemoral (subiliac) lymph node is a superficial node located laterally on the cranial border of the tensor fasciae latae, half the distance between the coxal tuber and the patella. It is laterally bounded by the deep surface of the cutaneous muscle (panniculus carvanosus). The node drains the skin and subcutis of the pelvic and cranial part of the thigh area as well as the skin and subcutis caudal to the transverse line drawn through the seventh or eighth rib, except for the caudoventral regions of the abdomen (Grau, 1933; cited in Sisson and Grossman, 1975; Figure 3.5a). The ducts accompany the circumflex iliac blood vessels on the deep surface of the tensor fasciae latae and then enter the abdominal cavity where they drain into the lateral or medial iliac lymph nodes or join the lumbar lymphatic ducts directly (Figure 3.5b).

FIGURE 3.4 Comparison of cell separation techniques. PBLs were separated by either hypotonic lysis of RBCs using Tris-ammonium chloride or by density gradient centrifugation over lymphoprep and stained for flow cytometry using SBU-T1, SBU-T4, SBU-T8, SBU-T19 and VPM 30 in order to assess the proportion of $CD5^+$, $CD4^+$, $CD8^+$, $\gamma\delta$ and B cells stained by each antibody in the cell populations.

a(——) = cells separated by Tris-NH_4Cl lysis
b(.....) = cells separated by lymphoprep
c(. . . .) = Negative control

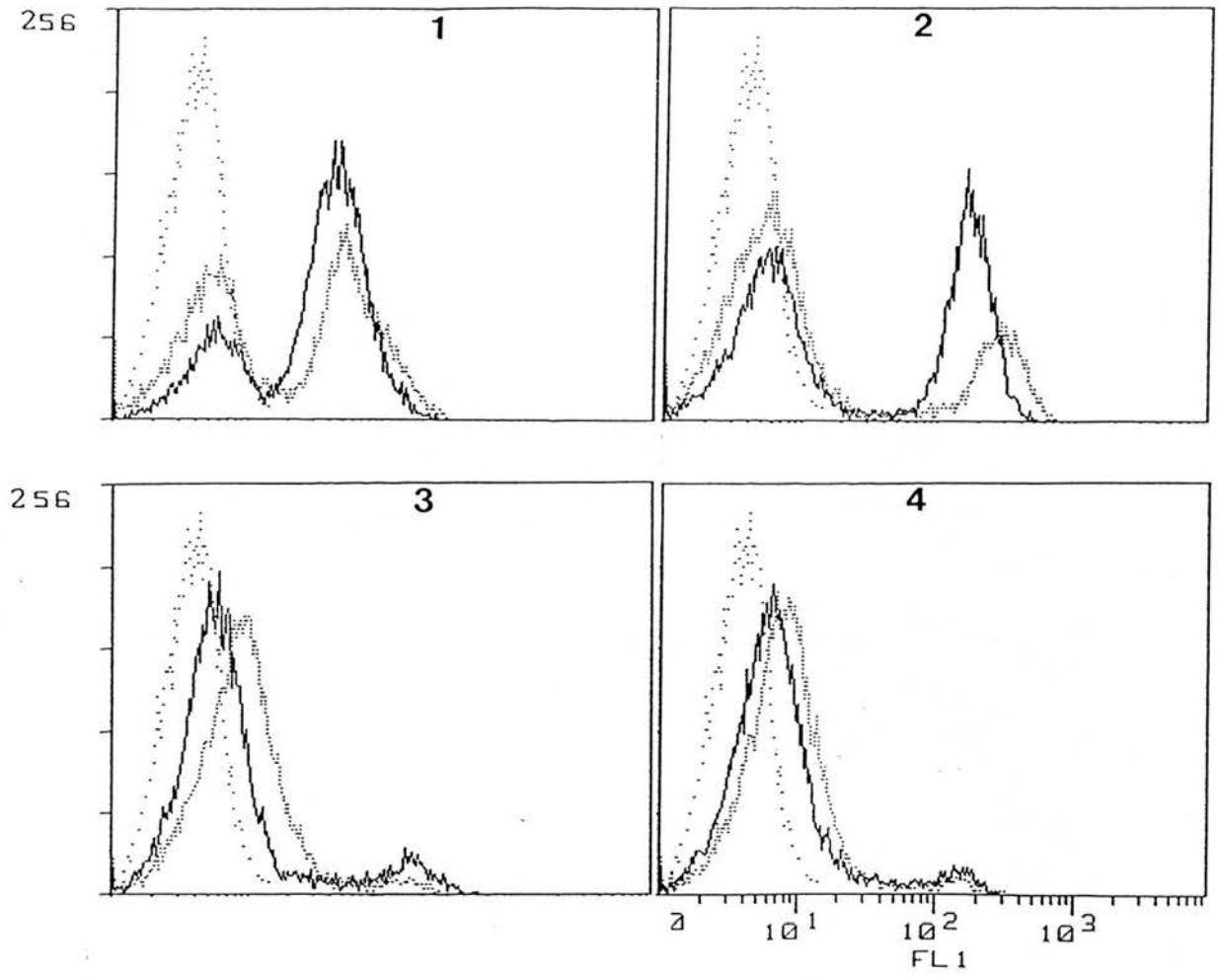
1. $CD5^+$ cells
(a) Lysis = 68.8 percent positive
(b) Lymphoprep = 51.8 percent positive

2. $CD4^+$ cells
(a) Lysis = 47.8 percent positive
(b) Lymphoprep = 25.9 percent positive

3. $CD8^+$ cells
(a) Lysis = 15.3 percent positive
(b) Lymphoprep = 13.0 percent positive

4. $\gamma\delta^+$ T cells
(a) Lysis = 10.1 percent positive
(b) Lymphoprep = 8.8 percent positive

5. B cells
(a) Lysis = 42.8 percent positive
(b) Lymphoprep = 57.1 percent positive


256
1
2
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3
4
0
10^0
10^1
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10^3
FL1
256
5
0
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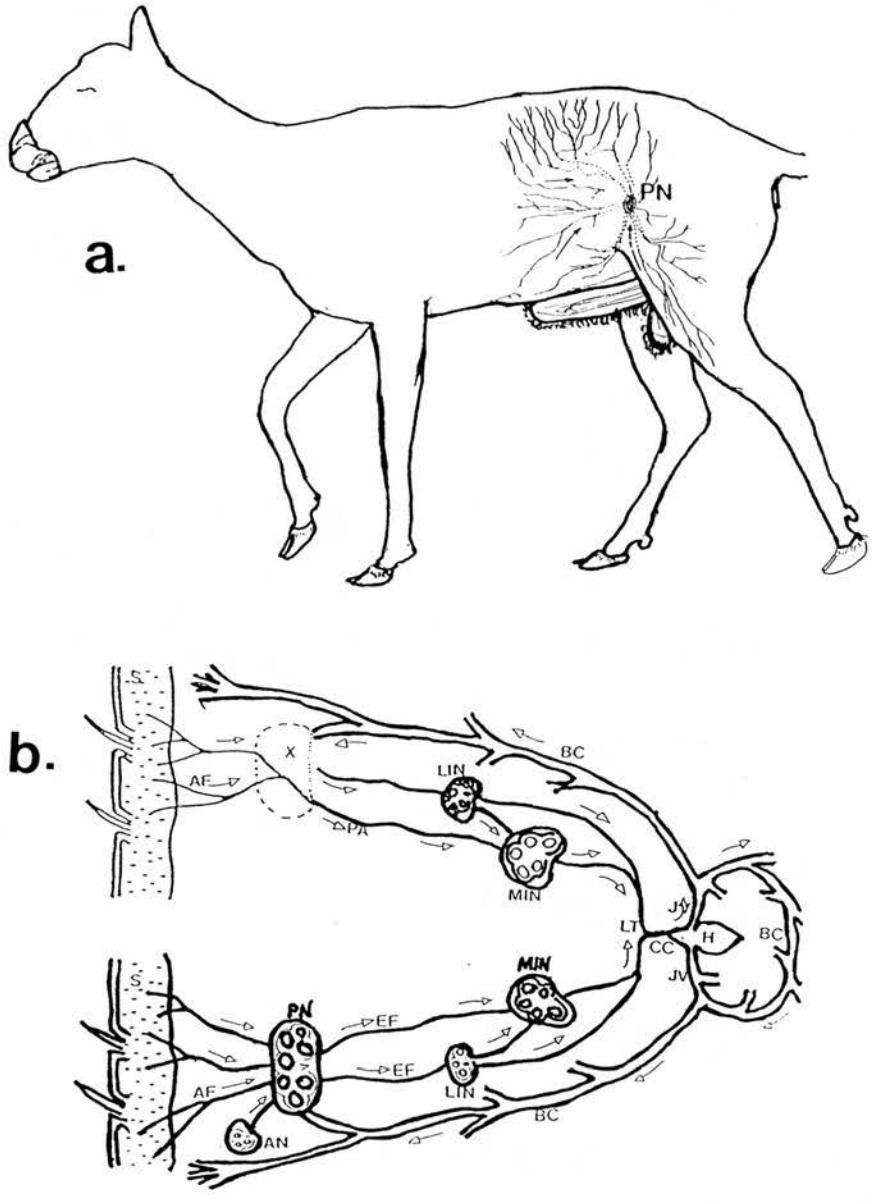
10^1
10^2
10^3
FL1

Table 3.2 **Optimal working dilutions for monoclonal antibodies and conjugates for indirect immunofluorescence**

	Dilution
SBU-T1	1:500
SBU-T4	1:500
SBU-T8	1:500
SBU-T19	1:250
SBU-T6	1:250
SBU-I	1:500
SBU-II	1:500
SBU-LCA	1:500
SBU-p220	1:500
NMS	1:1000
Anti-metacyclic mAb	1:1000
$F(ab')_2$RAS Ig	1:50
SBU-T4 biotin	1:50
SBU-T8 biotin	1:50
SBU-T19 biotin	1:50
VPM 30 biotin	1:50
GAR/Ig/FITC	1:80
SHAM/IgG_1/FITC	1:150
SHAM/IgG_{2a}/FITC	1:250
SHAM/IgM/FITC	1:250
PE-SA complex	1:250
NMS-biotin	1:250



E 3.5 (a) Diagram showing the areas drained by afferent lymphatics of prefemoral lymph node (adapted from Sisson and Grossman's. The Anatomy of the Domestic Animals, Vol. 1, Editors R. Getty, W.B. Saunders Company, Philadelphia, London, Toronto, 1975).

(b) Schematic diagram showing lymph flow from areas drained by prefemoral node in sheep and indicating the reanastomosis of afferent lymphatics after lymphadenectomy. S, Skin; AF, Afferent lymphatic duct; AN, Accessory lymph node; PN, Prefemoral lymph node; X, Position of ablated lymph node; EF, Efferent lymphatic duct; PA, Pseudo-afferent lymphatic duct; LIN, Lateral iliac lymph node; MIN, Medial iliac lymph node, LT, Lymphatic trunk; CC, Cysterna chyli; H, Heart; JV, Jugular vein; BC Blood Circulation (adapted from Sissons, Grossman, 1975 and Akol, 1985).

3.6.1 Presurgical Procedures and Anaesthesia

Animals were starved overnight prior to surgery. Induction of anaesthesia was by intravenous Saffan™ (alphaxolone/alphadolone acetate; Pitman-More, U.K.) at 2 mg kg^{-1} and was maintained using gaseous halothane (Halothane BP, Dagenham, England) and 50:50 oxygen/nitrous oxide flow introduced using cuffed endotracheal tube. A closed circuit anaesthetic machine, Fluotec 3 (Boyles International 2, Harlow, England) fitted with a CO_2 absorber and rebreathing bag, was used to deliver the halothane/O_2/CO_2 gases.

3.6.2 Surgical Procedure

Cannulation of the pefemoral efferent lymphatic was as described by Hall and Morris (1965). Strict asceptic technique was used throughout. The duct was cannulated using a Portex polypropylene cannula which was exteriorised via a stab wound. The cannulation wound was closed with a continuous 2/0 catgut (Ethicon Ltd, Edinburgh, Scotland) followed by Michell-clips. Immediately after the operation the sheep were given 5 mls of Depomycin forte (procaine-penicillin/ dihydro-streptomycin sulphate; Mycofarm, U.K.) at a site distal to the cannulation and as well 1 ml (1000 I.U.) heparin subcutaneously within the prefemoral drainage area to obviate immediate postsurgical clotting.

Lymph was collected into sterile, siliconised plastic bottles containing 1000 I.U. heparin daily at 10.00 am. The volume was determined using a measuring cylinder in order to calculate the hourly flow rate. The cells were enumerated using a System 9000 automated cell counter. The cell counts per ml and the flow rates were used to calculate the total and hourly cell outputs. In all cases, cannulations were allowed 5 days to stabilise before collecting lymph for analysis.

3.6.3 Preparation of Efferent Lymphocytes

25 mls of lymph were centrifuged at 800 g for 15 minutes at 4°C to separate cells from lymph fluid. The cell-free lymph was decanted and stored at -20°C until used. The cell pellet was then resuspended in IFB and contaminating erythrocytes lysed with tris-ammonium chloride where necessary.

3.7 Quantitation of Parasite Specific Serum Antibody

3.7.1 Serum Sample Preparation

Serum samples were prepared from jugular blood. 10 mls of blood were collected in glass universal bottles, and allowed to clot in an inclined position in a 37°C incubation for 30 minutes. Alternatively the samples were left overnight at

4°C. The formed serum was pipetted into plastic universal bottles and spun at 10,000 g for 20 minutes at 4°C. 5 mls of the supernatant were collected and stored at -20°C until used.

3.7.2 Preparation of Trypanosomal Antigen

The antigen was prepared from purified *T. evansi* TREU 2143. Infected blood was collected from mice with fulminating parasitaemia and parasites separated on a column of DE-52 as described in Appendix III(1). The trypanosomes were resuspended in 2 mls of PBS and subjected to two cycles of freeze-thawing at -70°C. The sample was then diluted 1/4 with ice-cold PBS, kept refrigerated in an ice bath and subjected to four cycles of ultrasonication for 45 seconds at maximum amplitude on an MSE 100 W Ultrasonic Disintegrator. The resulting suspension was centrifuged at 10,000 g for 20 minutes to remove debris. The protein concentration of the supernate was then determined photometrically, adjusted to 1 mg ml^{-1}, aliquoted and frozen at -20°C as stock antigen.

3.7.3 MicroELISA Technique

T. evansi-specific antibody responses were assayed using the micro-scale enzyme-linked immunosorbent assay (microELISA) as described by Luckins (1977) and modified by Payne *et al.* (1988). Specific IgG1 and IgM were detected in an indirect (double sandwich) microELISA using mouse anti-sheep IgG1 and IgM mAbs (kind gifts from Dr K.J. Beh, C.S.I.R.O., Division of Animal Health, McMaster Laboratory, Glebe, Australia) and horseradish peroxidase (HRP) conjugated goat anti-mouse polyvalent immunoglobulins (SIGMA). Detailed protocol of the ELISA technique used is given in Appendix III(4). The mAbs and immunoconjugates used in all ELISA systems are listed in Tables 3.1a and b respectively.

3.7.4 Optimisation of Antigen, Reference Sera and Conjugate Dilutions

The optimal working dilutions of trypanosomal antigen was determined empirically in an ELISA. Doubling dilutions of the antigen from 1:25 to 1:1600 in 0.05 M carbonate/bicarbonate buffer, pH 9.6 (Appendix II(6) were made. Four replicates of each of the dilutions were tested against a 1:500 arbitrary dilution of the negative and positive reference sera and 1:300 dilution of DASh/HRP/Ig (SAPU, Scotland). The negative reference serum was obtained from 2-week old specific-pathogen-free lambs (courtesy, Dr W. Donachie, Moredun Institute, Edinburgh) while the positive reference serum was a 21 days p.i. sample from a Suffolk lamb infected with 2×10^6 *T. evansi* TREU 2143. The 1:100 dilution representing an antigen concentration of 10 µg ml^{-1} was chosen since it gave the greatest difference

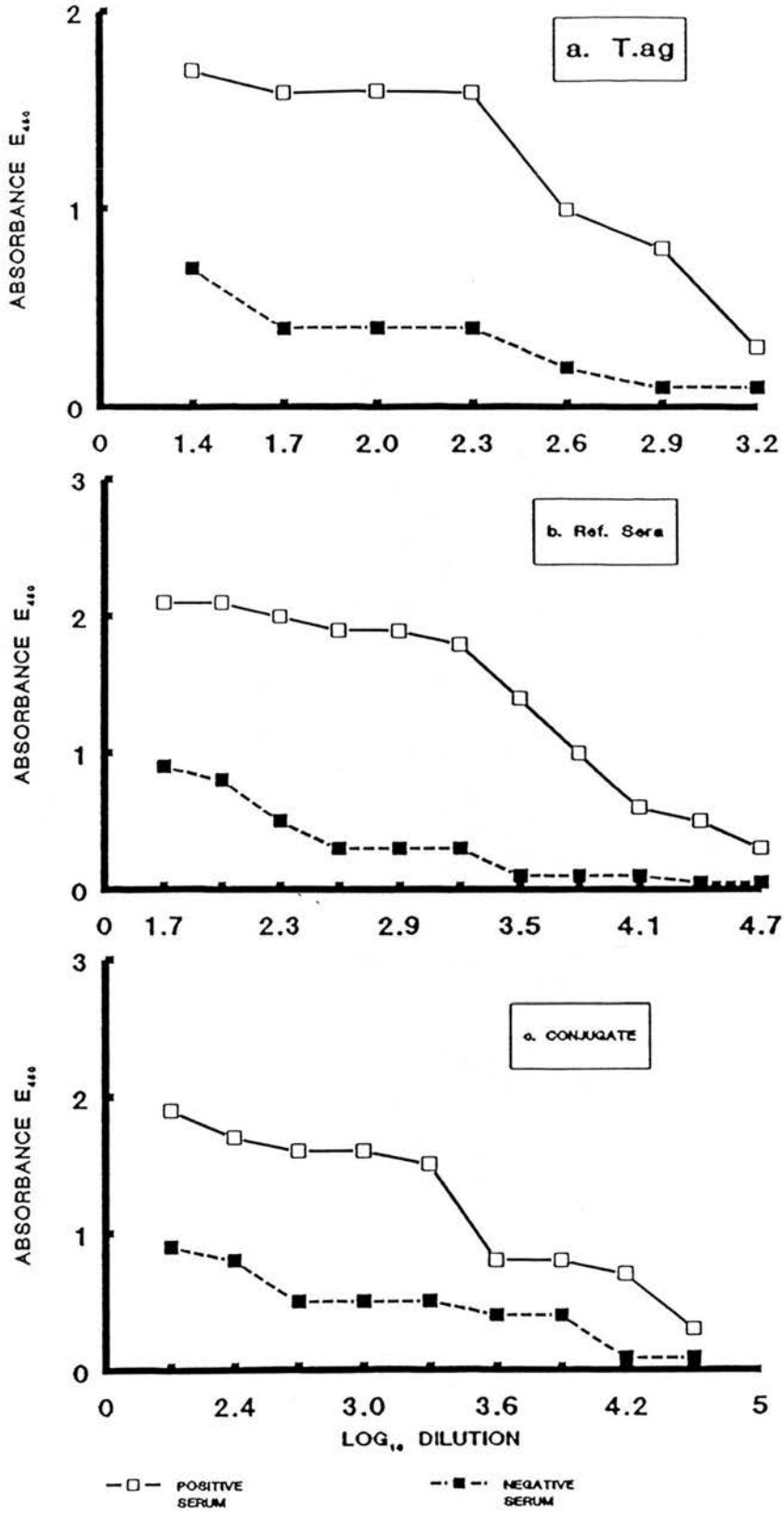


FIGURE 3.6 I. Determination of optimal dilutions of trypanosomal antigen, positive and negative reference sera for trypanosome specific antibody ELISA.

(a) Trypanosomal antigen titration
(b) Negative and positive reference sera titration
(c) Conjugate titration

between the optical density values of the negative and positive reference sera (Figure 3.6a).

The reference sera were tested in dilutions of 1:50 to 1:51200 against the optimal antigen dilution and an arbitrary conjugate dilution of 1:300. The 1:800 dilution gave the greatest difference between the two (Figure 3.6b) and was used throughout the work. The conjugate was tested as dilutions of 1:125 to 1:32000 against the optimal antigen and reference sera dilutions with the 1:1000 chosen as the optimal working dilution (Figure 3.6c).

3.8 Measurement of Immune Responses to *Pasteurella* Vaccine

3.8.1 Vaccination of Sheep

A monovalent *Pasteurella haemolytica* A1 vaccine prepared from its sodium salicylate capsular extract (SSE) and adsorbed onto aluminium hydroxide gel (alhydrogel) (Gilmour *et al.*, 1983; Fodor and Donachie, 1988) was used in the study. Sheep were primed and boosted by subcutaneous inoculation of 2 mg doses of the vaccine four weeks apart except in efferent lymphatic duct cannulation experiments where they were administered 10-14 days apart. Prior to vaccination the site was clipped and disinfected with 70 percent alcohol.

3.8.2 Assessment of Local Skin Reaction to Vaccine Administration

Local inflammatory reaction at the site of vaccine administration was assessed by visual examination, touch and by the measurement of the skin thickness using a pair of vernier calipers.

3.8.3 Quantitation of *Pasteurella*-specific antibodies

Antibody responses to *Pasteurella* vaccination were assayed in both direct and double sandwich microELISA techniques using mouse anti-sheep IgG_1, IgG_2 and IgM mAbs (Table 3.1a). The ELISA protocol is given in Appendix III(4). The conjugates are listed in Table 3.1b.

3.8.3.1 *Pasteurella* Antigen, Positive and Negative Serum

The *Pasteurella haemolytica* A1 antigen as well as the negative and positive reference sera used in the ELISA were gifts from Dr W. Donachie. The antigen was supplied as a 10 mg ml^{-1} SSE suspension but was later adjusted to 2 mg ml^{-1}, aliquoted and stored at -20°C until used. The positive reference serum was obtained from SPF lambs vaccinated at 3 weeks of age and boosted 4 weeks later with the A1 SSE vaccine (Fodor and Donachie, 1988). The negative reference serum was obtained from a two week old unvaccinated SPF lamb.

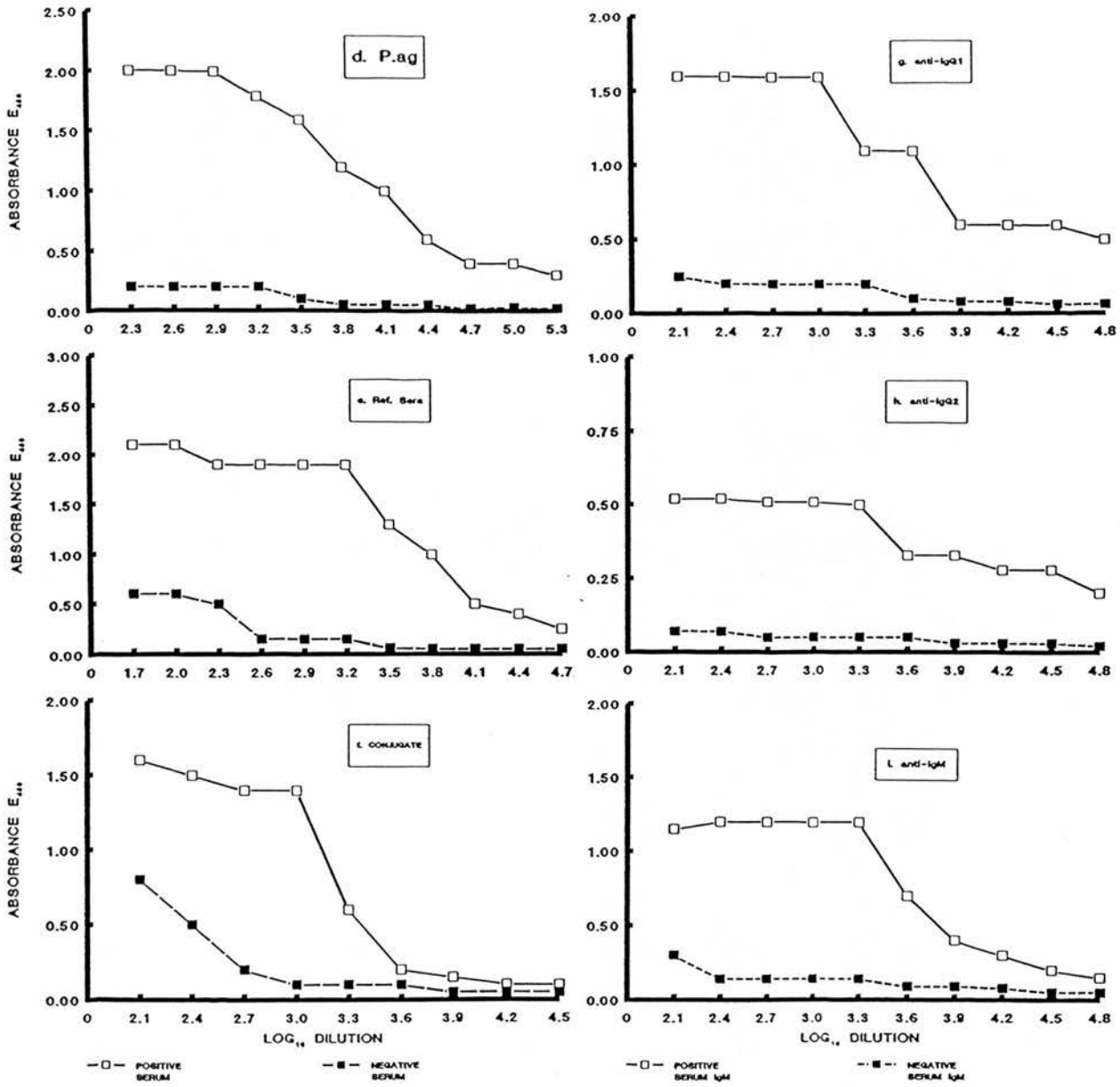


FIGURE 3.6 II. Determination of optimal working dilutions of *Pasteurella* antigen, positive and negative reference sera, conjugates and mAbs for *Pasteurella*-specific antibody ELISA.

3.8.3.2 Test Serum and Lymph Preparation

Test serum was prepared as described in section 3.8.1 earlier. Lymph was collected from cannulated sheep and cell-free lymph prepared as described in Section 3.7.4. Before use, the serum was spun at 10,000 g for 20 minutes to remove debris.

3.8.3.3 Optimising *Pasteurella* Antigen, Reference Sera, mAbs and Conjugate Dilutions

The antigen, reference sera and conjugates were titrated in an ELISA as described by Burrells *et al.* (1979) and Donachie *et al.* (1983). The antigen was diluted 1:200 to 1:204800 and tested against arbitrary 1:100 and 1:500 dilutions of the reference sera and conjugate respectively. There was no difference between the values for 1:200 to 1:800 (Figure 3.6d) so the median point of 1:400 (5 $\mu g\ ml^{-1}$ antigen concentration) was chosen as the optimal antigen dilution. The reference sera were titrated against the conjugate and optimal antigen dilution. The 1:800 dilution (Figure 3.6e) gave the optimal response. Figures 3.6f and 3.6g-i show the results of the titrations of the conjugates and the mAbs respectively. The 1:1000 dilution was optimal for the conjugates and the antibodies respectively.

3.9 *In vitro* Functional Assay of Sheep Peripheral Blood Leucocytes

The functional status of lymphocytes from sheep following infection with *T. evansi* TREU 2143 and/or inoculation with *Pasteurella haemolytica* vaccine was assayed in *in vitro* culture systems. Their proliferative responses upon stimulation with mitogens or specific *Pasteurella* and trypanosomal antigens were used as the index of functional activity. Furthermore, cell depletion experiments were conducted in order to assess the role of the depleted cell population. The general protocol of the *in vitro* culture system used is given in Appendix III(5).

3.9.1 Mitogens and Antigens for Lymphoproliferative Assays

Two mitogens, concanavalin A (Con A) from *Conavalia ensiformis* (Jack Bean) type V, and bacterial cell wall lipopolysaccharide (LPS) from *E. coli* 055:B5, were purchased from Sigma Chemical Co., St Louis, Mo, U.S.A. and used in the study. *Pasteurella haemolytica* A1 and *T. evansi* TREU 2143 were used as the specific antigens .

Con A is a lectin with mitogenic specificity for T lymphocytes. It was reconstituted to a concentration of 250 $\mu g\ ml^{-1}$ (w/v) using RPM1 1640 complete culture medium (GIBCO Ltd, U.K., Appendix II(7)). This was aliquotted and stored at -20^{o}C as stock mitogen. Prior to use, the stock was adjusted to a concentration

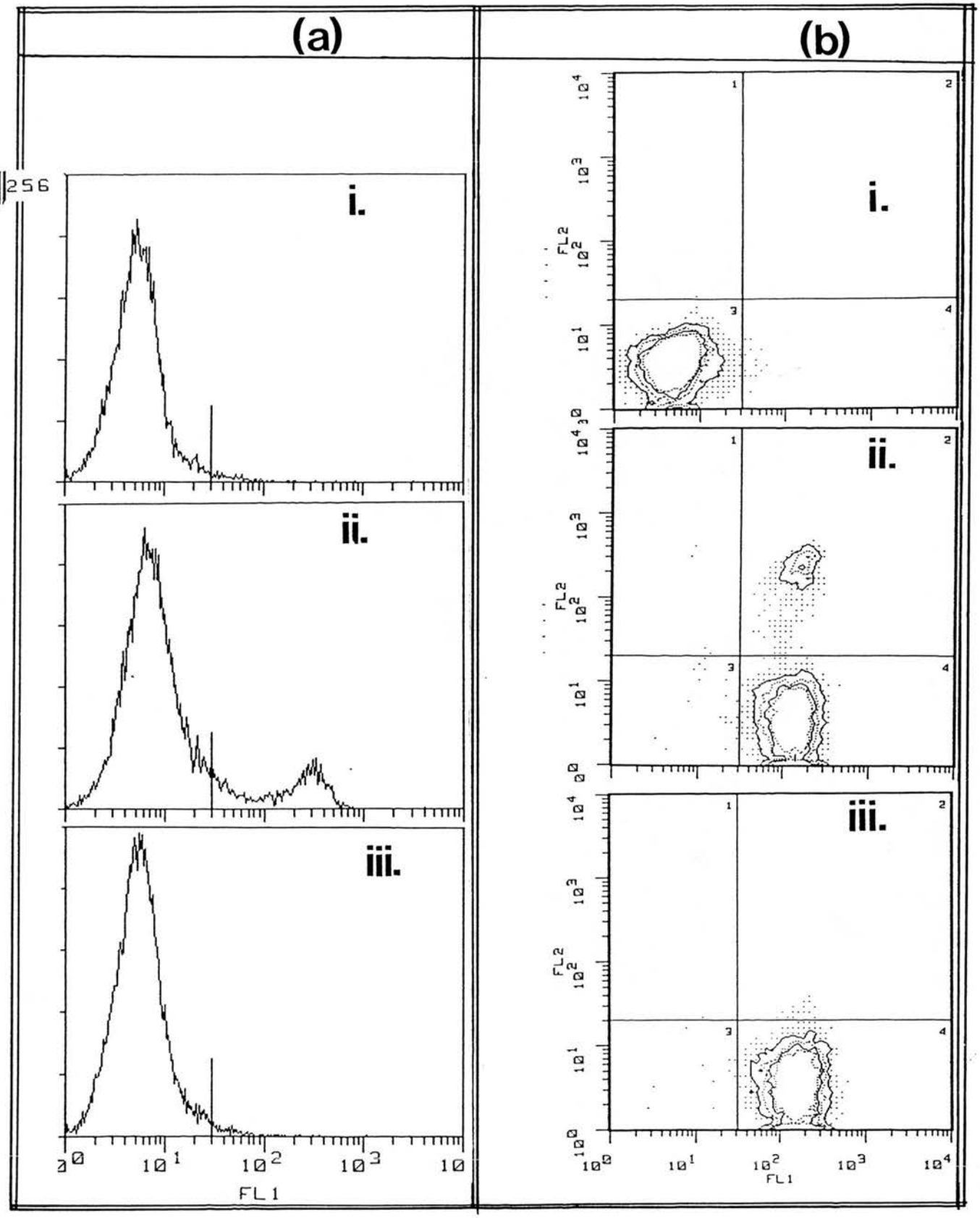


FIGURE 3.7 Test of the efficiency of $CD8^+$ T cell depletion by monoclonal anti-CD8 (S-T8) treatment plus complement.

(a) Depleted and undepleted cell populations were subjected to single colour immunofluorescence staining using S-T8 and then analysed by flow cytometry

- i. Frequency histogram of the negative control
- ii. Frequency histogram of $CD8^+$ cells from the undepleted cells
- iii. Frequency histogram of $CD8^+$ cell from the depleted population. More than 98 percent depletion of the $CD8^+$ cell population was consistently obtained.

(b) Depleted and undepleted cells were also subjected to two-colour flow cytometry using biotinylated SBU-T8 and SBU I (MHC I).

- i. Contour profile of the negative control
- ii. Contour profile of the undepleted cells

Quadrant	1	=	$CD8^+CL1^-$ cells (none)
-	2	=	$CD8^+CL1^+$ cells
-	3	=	$CD8^-CL1^-$ cells (none)
-	4	=	$CD8^-CL^+$ cells

- iii. Contour profile of the depleted cells

Quadrant	1	=	as above
-	2	=	" " (none)
-	3	=	" "
-	4	=	" "

of 50 μg ml^{-1} as the working stock. LPS is a hghly immunogenic constituent of the cell wall of most gram negative bacteria. It has an adjuvant activity (Landy and Baker, 1966; Skidmore *et al.*, 1975) and also acts as a specific mitogen for B lymphocytes (Andersson, Moller and Sjoberg, 1972; Hammarstrom *et al.*, 1976; Smith *et al.*, 1979). It was supplied as a gamma-irradiated, lyophilised powder which was reconstituted in culture medium, adjusted to a concentration of 250 μg ml^{-1} and stored at -20°C until required.

P. haemolytica antigen was obtained as described under subsection 3.8.2. It was also adjusted with culture medium to a concentration of 250 μg ml^{-1} and stored at -20°C. *T. evansi* was used as either live or ultrasonicated antigen. Live trypanosomes were obtained aseptically using anion-exchange chromatography as described in Appendix III(1), and used as a stock adjusted to 10^7 ml^{-1} with complete culture medium. The ultrasonicated antigen prepared as described under subsection 3.7.2 was adjusted with complete culture medium to 250 μg ml^{-1} and sterilised again by ultrafiltration first through a 0.45 mm millipore then through a 0.22 mm Millipore filter.

3.9.2 Preparation of Cells for *in vitro* Functional Assays

3.9.2.1 Separation of Peripheral Blood Leucocytes

Sheep peripheral blood leucocytes were separated by density gradient centrifugation over lymphoprep (Appendix III(2). All washing processes were carried out using tissue culture wash medium (Appendix II(8) and, the cell concentrations adjusted to 10^7 ml^{-1} using complete culture medium.

3.9.2.2 Depletion of $CD8^+$ T Cells

$CD8^+$ T cells were depleted by antibody-mediated complement lysis (Tarleton, 1988b; Stefanovic *et al.*, 1989). Two mls of purified PBLs adjusted to 10^7 cells ml^{-1} in complete culture medium were incubated with 2 mls of culture supernatant fluid of the anti-CD8 mAb, S-T8 for 30 minutes at 4°C. The cells were then washed three times in tissue culture wash medium, resuspended in 1 ml of 1:10 dilution of rabbit complement (Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, England) and incubated at 37°C for 1 hour. Treated cells were then washed three times, counted and readjusted to 10^7 cells ml^{-1} in complete culture medium. Nigrosan dye exclusion test of the treated cells consistently gave >98 percent viable cell count. The efficiency of $CD8^+$ T cell depletion was assessed in a single and two-colour indirect immunofluorescence and flow cytometry using SBU-I, S-T8 and biotinylated SBU-T8 mAbs. The results are shown in Figures 3.7a and b.

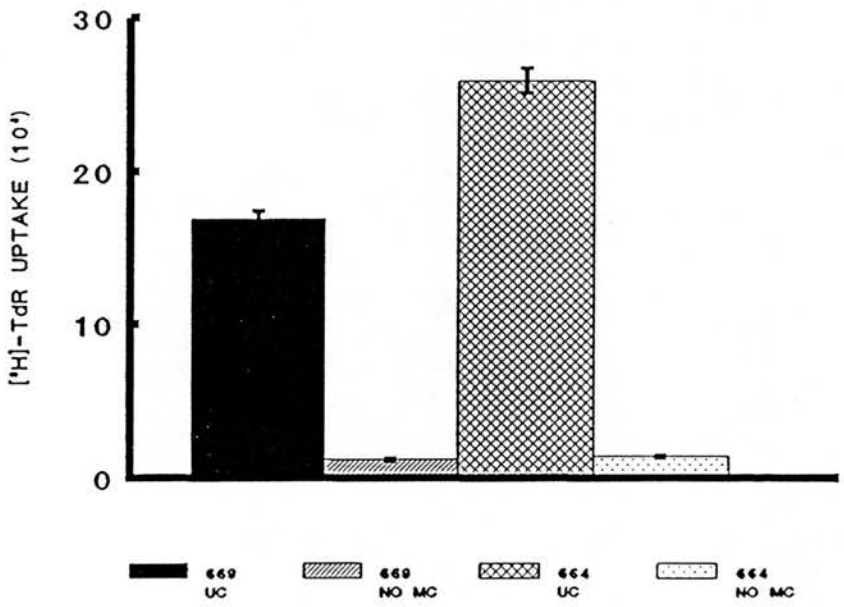


FIGURE 3.8 Testing the efficiency of monocyte depletion from PBLs.

Two sheep were primed and boosted with 2 mg doses of *P. haemolytica* vaccine 4 weeks apart. 6 weeks after the booster vaccination, PBLs separated from the jugular vein blood of the sheep were depleted of monocytes as described in Section 3.9.2.3. Five replicates each of 5 x 10^5 cells ml^{-1} from the depleted and undepleted cell population were subjected to specific stimulation with *Pasteurella* antigen in an *in vitro* culture system. In both sheep, depleted cells failed to respond to the antigen.

3.9.2.3 Removal of Monocytes

Monocytes were removed from the cell population by incubating 5 x 10^7 PBLs suspended in 10 mls of culture medium for 1 hour at 37°C in a humid atmosphere of 5 percent CO_2/95 percent air using a 25 ml Falcon tissue culture flask (Scientific Supplies, Co., Vine Hill, London). The nonadherent cells were removed by vigorous swirling with warm (37°C) culture medium and recultured twice in fresh flasks under the same conditions and duration as above. The final suspension of nonadherent cells were then washed twice in wash medium, counted and readjusted to the working concentration of 10^7 cells ml^{-1}. The efficiency of removal of monocytes was tested by comparing the responses of depleted and nondepleted cell populations to *in vitro* stimulation with *P. haemolytica* antigen. These cells were obtained from sheep that had previously been primed and subsequently boosted with 2 mg doses of *P. haemolytica* vaccine. Figure 3.8 shows failure of the adherent cell depleted population to respond to specific antigen *in vitro*.

3.9.2.4 Depletion of $CD8^+$ T Cells and Monocytes

$CD8^+$ T cells and monocytes were removed by first subjecting 5 x 10^7 PBL ml^{-1} to monocyte depletion, followed by antibody and complement mediated cytotoxic killing of $CD8^+$ T cells as described above. The resulting cell suspension was washed, counted and readjusted to 10^7 cells ml^{-1} with complete culture medium.

3.9.3 Optimisation of Culture System Conditions

3.9.3.1 Cell Concentration

Six different cell concentrations were used to determine the number of cells which gave optimal proliferative responses to previously optimised concentrations of Con A, LPS, and P. ag. The cells were dispensed into 96-well flat-bottomed NUNC microculture plates (Flow Laboratories, Rickmansworth, Herts,U.K.) in triplicate in volumes of 2.5, 5, 10, 20, 40 and 80 µls, to give final cell concentrations of 2.5 x 10^4, 5 x 10^4, 10^5, 2 x 10^5, 4 x 10^5 and 8 x 10^5 per well respectively. From stock solutions of 50 µg ml^{-1} Con A and 250 µg ml^{-1} LPS and *Pasteurella* antigen (P. ag.), respective volumes of 10 µls, 60 µls and 40 µls were added to the cells. Complete culture medium was then added so that each well contained a total volume of 200 µls. The cultures were incubated at 37°C for 72 hours in a humified atmosphere of 5 percent CO_2/95 percent air. Cells were then pulsed with 0.5 µCi of [methyl-^{3}H] thymidine (specific activity 5Ci $mMol^{-1}$; Amersham, Amersham International plc, Buckinghamshire, England) during the last 5 hours of culture. The cells were harvested onto glass fibre filter paper discs

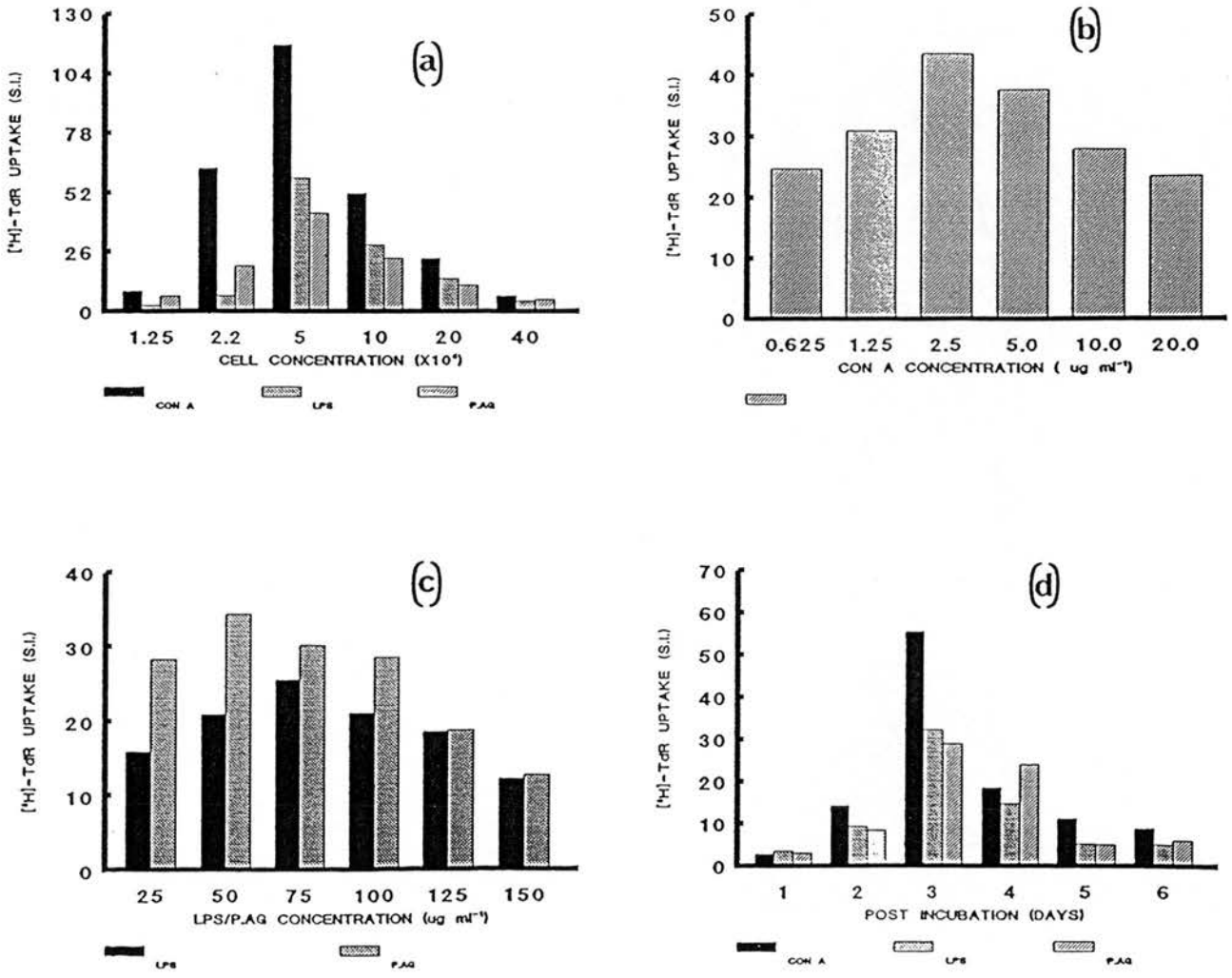


FIGURE 3.9 Optimisation of culture system variables for *in vitro* lymphocyte transformation assays.

(a) Titration for determining optimal cell concentration
(b) Titration for determining optimal Con A concentration
(c) Titration for determining optimal LPS and *Pasteurella* antigen concentrations.
(d) Time curve (optimal duration of culture)

(Whatman, U.K.) using a semi-automated cell harvester, multi MASH II (Dynatech). The filter strips were dried for 30 minutes at 65°C and the discs placed in disposable plastic scintillation vials (Sterilin Ltd, Middlesex). 2 mls of scintillation fluid were dispensed into each vial, the vials were then capped and thymidine incorporation was determined by counting in a beta liquid scintillation spectrometer (Packard 2000).

The optimum cell concentration is regarded as that which gives the highest cell count per minute (CPM) of the treated cells and the lowest CPM of the untreated cell, which is usually reflected in higher stimulation index. For both Con A and LPS as well as P. ag the cell number which gave maximum CPM with low background and blastogenesis was 10^5 cells well^{-1} (Figure 3.9a). This represents a final cell concentration of 5 x 10^5 cells ml^{-1} and was used throughout the assay.

3.9.3.2 Mitogen and *Pasteurella* Antigen Concentrations

Triplicate cultures were set up as above using an arbitrary cell concentration of 2 x 10^5 in 20 µl volumes. A range of concentrations of Con A, LPS and P. ag were titrated against the cell. A triplicate control consisting of cell culture without mitogen or antigen added was also set up. Figure 3.9b and c shows that the optimal concentration as determined by CPM and SI for Con A was 0.5 µg well^{-1}, representing a final concentration of 2.5 µg ml^{-1}; for LPS was 15 µg well^{-1} or 75 µg ml^{-1} final dilution and, for P. ag was 10 µg well^{-1} representing a final concentration of 50 µg ml^{-1}.

3.9.3.3 Time Curve

The time of optimal blastogenesis in an equilibrated system was determined by setting up cultures for Con A, LPS and P. ag in quadruplicate. Six plates were used per mitogen or antigen and one each harvested and counted daily as already described. From Figure 3.9d the 72 hour (3 days) culture gave the best CPM and SI for all the systems, hence 72 hours was chosen as the optimal culture duration.

3.9.3.4 Trypanosome Concentration and Survival

To determine the optimal survival time of trypanosomes in culture without daily changes of culture medium, a range of parasite concentrations from 1.25 x 10^5 ml^{-1} to 8 x 10^6 ml^{-1} were set up in culture and counted daily. Each concentration was used to set up cultures in 12 wells in a total culture medium volume of 200 µls. Two wells of each dilution were washed out with 1 ml of culture medium daily and counted. Table 3.3 shows that parasite survival time was

maximal at a concentration of 5 x 10^5 ml^{-1}.

Table 3.3 **Parasite concentrations and numbers surviving daily over 6 days of culture without daily change of culture medium**

	Parasite numbers (x10^5 ml^{-1})						
Days	0	1	2	3	4	5	6
	1.25	1.3	1.25	1.00	0.50	0.02	0.001
	2.50	2.55	2.50	1.75	0.75	0.02	0.001
	5.00	5.15	5.18	4.50	1.20	0.04	0.001
	10.0	8.20	6.00	2.90	0.80	0.002	0
	20.0	12.50	5.51	2.10	0.40	0.002	0
	40.0	14.45	3.10	0.55	0.10	0	0
	80.0	20.15	2.00	1.01	0.001	0	0

3.9.4 Test Culture Systems

The above optimised *in vitro* system was used to study the proliferative responses of PBLs from sheep infected with *T. evansi* and also vaccinated against *Pasteurella* or had been infected and treated with a trypanocidal drug. Cells were also taken from non-infected, *Pasteurella* vaccinated sheep. In addition, cells from these categories of sheep were taken and selectively depleted of $CD8^+$ cells and monocytes as already described and used in the culture system. The purified cells were then used to set up cultures in triplicates as described in Appendix III(5) as follows:

For animals with active infection and/or vaccinated against *Pasteurella*

1. Medium alone	(200 µls)
2. Cells + medium	"
3. Cells + Con A	"
4. Cells + Con A + Trypanosomal antigen (T. ag)	"
5. Cells + LPS	"
6. Cells + LPS + T. ag.	"
	(200 µls)

7. Cells + P. ag "
8. Cells + P. ag + T. ag "
9. Cells + T. ag. "
10. Con A or LPS + T. ag. "

For cell depletion cultures

1. Cells + medium
2. Cells depleted of monocytes + Con A or LPS
3. Cells depleted of $CD8^+$ cells + Con A or LPS or P. ag or T. ag
4. Cells depleted of $CD8^+$ cells and monocytes + Con A or LPS
5. Non depleted cells + Con A or LPS or P. ag or T. ag

The results were presented either as counts per minute (CPM) or as stimulation indices (SI).

CHAPTER FOUR

Peripheral Blood Leucocyte Phenotype and Antibody Responses in Ovine *T. evansi* Trypanosomosis

4.1 Introduction

The examination of peripheral blood leucocyte subset demographics along with parasite-specific antibody responses is an approach which might lead to a better understanding of the complex immunological events involved in determining trypanotolerance or trypanosusceptibility. This is because lymphocytes, continuously enter and exit lymphoid and nonlymphoid organs using the blood as a traffic route, with a mean transit time of approximately 30 hours (Westermann and Pabst, 1990). This means that roughly 5×10^{11} lymphocytes travel through the blood each day (Matsuda, Uchida and Kariyone, 1985; Pabst, 1988). Since this number is about the same as the entire lymphocytes within the system (Westermann and Pabst, 1990), the study of the dynamics and alterations in their phenotypes in the blood could provide useful information on the immunological status of the lymphoid system in general and on how they affect humoral responses during disease.

T. congolense infections in cattle and sheep are associated with dramatic changes in the dynamics of expression of various lymphocyte subsets in the periperhal blood (Ellis *et al.*, 1987; Mwangi, 1991; Williams *et al.*, 1991) as well as in the skin, afferent and efferent lymph and lymph node draining the site of infection with metacyclic forms of *T. congolense* (Mwangi *et al.*, 1990; 1991). Some of these lymphocyte subsets, such as $CD4^+$ and $CD8^+$ T cells, synthesise cytokines which in normal immunological responses enhance the proliferation and differentiation of B cells into specific antibody producing plasma cells (Kierszenbaum and Sztein, 1990; Pentreath, 1991). It is likely therefore, that alterations in the various T cell subsets resulting in an altered balance in lymphokine production (Sileghem *et al.*, 1989a) may underlie immunological aberrations in B cell function and failure to produce sterilising specific antibody isotypes during trypanosome infections.

The following experiments determine the dynamics of expression of various lymphocyte phenotypes in the peripheral blood during the course of trypanosome infection in sheep infected with a clone of *T. evansi* together with changes in routine haematology, clinical parameters and parasite specific antibody responses.

4.2 Materials and Methods

4.2.1 Experimental Animals and Trypanosome Infection

Eight yearling Finnish Landrace sheep of either sex were used in this study. Each sheep was infected by i.v. inoculation of 2×10^6 bloodstream trypomastigotes of *T. evansi* TREU 2143.

4.2.2 Experimental Design

The experiment was conducted as set out in Table 4.1 below.

Table 4.1

Sheep identification	Frequency of blood and serum sampling	Sample analysis	
		PBL subsets	Parasite-specific antibody
736 (female) 744 (female) 767 (male) 944 (female) 945 (male) 948 (female) 950 (male) 764 (female)	Day -13 to Day 0 weekly Day 1 to Day 61 twice weekly Day 62 to Day 99 weekly	By indirect immuno-fluorescence and flow cytometry	By microELISA

4.2.3 Clinical and Parasitological Parameters

The routine clinical parameters: body temperature, PCV, erythrocyte counts, total and differential leucocyte counts, and monitoring of parasitaemia were carried out as described in Section 3.3. The absolute numbers of the lymphocytes, neutrophils, monocytes and eosinophils were based on the product of the total leucocyte counts and their percentages out of 2000 cells counted in Giemsa stained thin blood film preparations.

4.2.4 Analysis of Lymphocyte Subsets

This was done by single colour indirect immunofluorescence staining of purified PBLs using a panel of 9 mAbs and a polyclonal F[ab'] rabbit anti-sheep (RAS) Ig followed by flow cytometry. The protocols for PBL isolation and staining have been detailed in Sections 3.4 and 3.5.3 respectively. Similarly, the characteristics of the mAbs and conjugates used have been itemised in Tables 3.1a and 3.1b. The results are presented as proportions of 10,000 cells analysed by flow cytometry and their absolute numbers per ml of peripheral blood computed on the basis of their proportions and the absolute numbers of the lymphocytes.

4.2.5 Assay of Parasite-Specific Antibody Responses

The IgG_1 and IgM responses during the course of *T. evansi* TREU 2143 infection in sheep were assayed in a microELISA technique as described in Section 3.8.4. Results were quantified photometrically using a microELISA minireader (Dynatech) at a wavelength of 450 nm and recorded as optical densities (O.D.).

4.3 Results

Two sheep, 950 and 764, selfcured at days 50 and 54 p.i. respectively and also showed similar trends in their responses. On this basis, some data are presented to show the responses of two groups. Group A comprises sheep, 736,

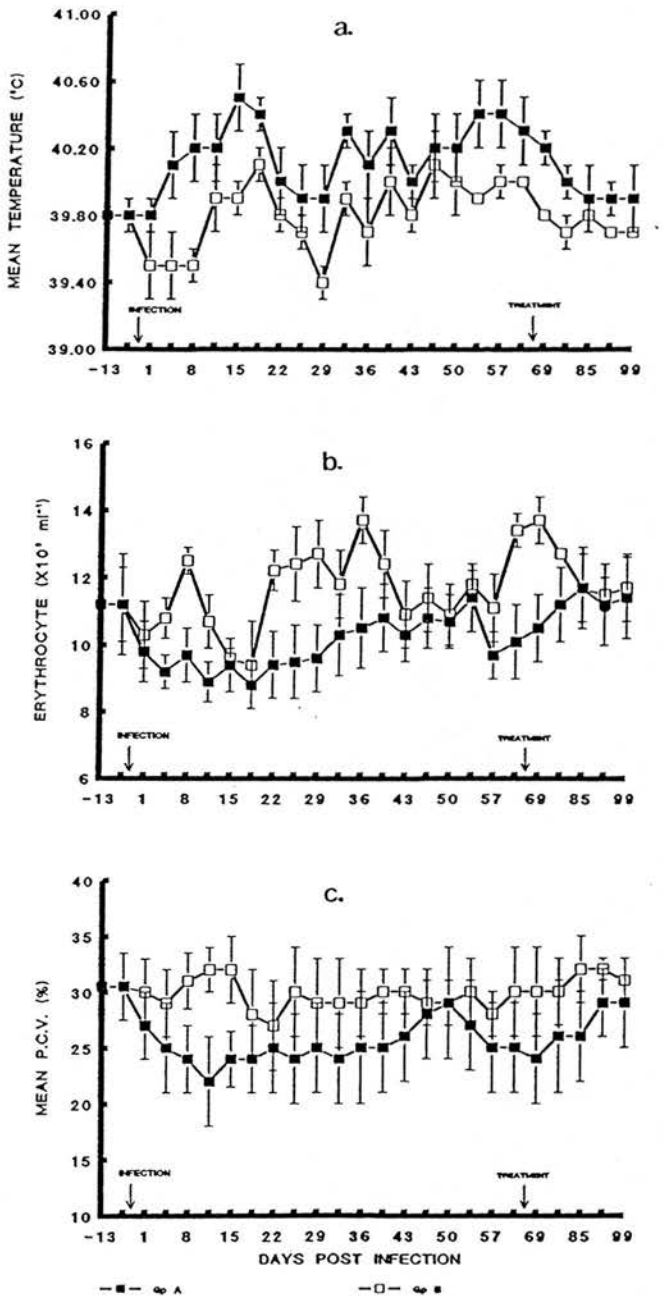


FIGURE 4.1 Mean changes in temperature, erythrocyte counts and packed cell volumes (PCV) in *T. evansi* infected sheep. Sheep in group B underwent selfcure on days 50 and 54 p.i. respectively

(a) Mean temperatures ± S.D.
(b) Mean erythrocyte counts ± S.D.
(c) Mean PCV ± S.D.

744, 767, 944, 945 and 948 which remained parasitaemic until treated and showed similar trends in their responses, while group B is made up of sheep 950 and 764 which required no drug therapy. The group A sheep were treated by i.v. injection of 10 mg kg^{-1} Naganol^R (Suramin, Bayer) on day 62 p.i, which failed to clear the parasites and was thus followed on day 74 p.i. by deep i.m. injection of Antrycide^R (Quinapyramine sulphate, B. Vet. C) at 5 mg kg^{-1} body weight. Confirmation of selfcure by group B and eventual drug cure in group A was based on the failure of mice subinoculated with blood samples to develop infection after 30 days of observation. The pooled data for all eight sheep are compared with values for day seven before infection by Student's paired t-test (Appendix v).

4.3.1 Clinical Assessment and Analysis of Routine Haematology

Infection was chronic in nature and characterised by low and frequently cryptic parasitaemias detected mostly by the microhaematocrit centrifugation technique. On only one occasion each in sheep 944, 948 and 950 on day 8, 13 and 18 p.i. respectively, was parasitaemia detected by the wet blood smear method. Despite this however, parasitaemia remained detectable in both groups by HCT until either selfcure or drug therapy. The onset of parasitaemia ranged from 8-15 days p.i. (Table 4.2). There were varying increases in the mean body temperature which fluctuated throughout the course of the infection but showed significant increases for the six sheep which remained parasitaemic ($p < 0.001$) by day 15 p.i. when compared with values 7 days prior to infection (Figure 4.1a). During the first four weeks of infection, the mean erythrocyte count of the six sheep showed a significant decrease ($p < 0.01$) from a preinfection mean of 11.2×10^6 ml^{-1} to 8.8×10^6 ml^{-1} by day 19 p.i. (Figure 4.1b). After day 29 p.i., although the erythrocyte counts remained below preinfection values until after drug treatment, they did not differ significantly ($p > 0.05$) from preinfection values. The mean PCV values for the six sheep also decreased significantly ($p < 0.001$) from a preinfection value of 31 percent to 22 percent by day 12 p.i. in the six sheep which were persistently infected, remaining throughout the course of the infection at or below 26 percent (Figure 4.1c). It is noteworthy however that in the two sheep which selfcured, apart from a decrease in erythrocyte count from a preinfection mean of 11.2×10^6 ml^{-1} to 9.6×10^6 ml^{-1} and 9.4×10^6 ml^{-1} on days 15 and 19 p.i. respectively, the mean erythrocyte counts remained within preinfection levels and in fact showed marked increases between days 22-40 p.i. (Figure 4.1b). Following selfcure, the mean

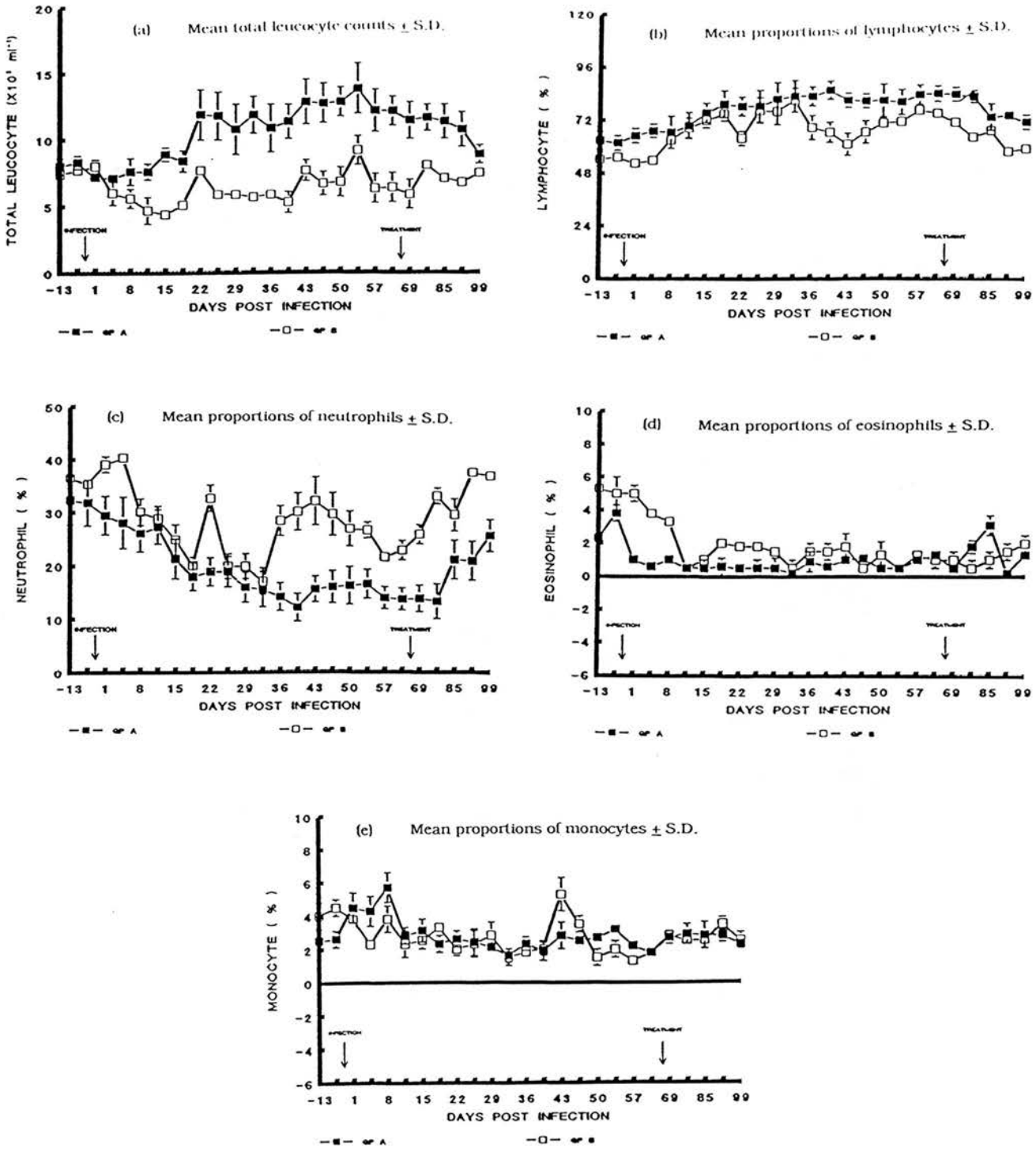


FIGURE 4.2 Changes in total and differential leucocyte counts in *T. evansi* infected sheep

Group B sheep selfcured on days 50 and 54 post infection.

erythrocyte counts of the group again rose above preinfection levels between days 61 to 78 p.i. There was no obvious change in mean PCV values (Figure 4.1c).

Table 4.2 **Days and methods of detection of first parasitaemia in sheep infected intravenously with *T. evansi* TREU 2143. HCT = microhaematocrit centrifugation technique; WM = Wet blood mount; p.i. = post infection.**

Sheep I.D.	Onset of Parasitaemia p.i.	Method of detection	Selfcure and day of
736	Day 12	HCT	No
744	" 9	HCT	No
767	" 11	HCT	No
944	" 9	WM	No
945	" 10	HCT	No
948	" 9	HCT	No
764	" 15	HCT	Yes/54
950	" 14	HCT	Yes/50

A transient initial decrease in the mean TWBC count of group B was followed by significant increases ($p < 0.05$) from preinfection levels by day 22 p.i. (Figure 4.2a). This rise was paralleled by a significant ($p < 0.001$) steady rise in the mean proportion (Figure 4.2b) and numbers (Table 4.3) of the lymphocytes and a steady decrease in the mean proportions and numbers of neutrophils (Figure 4.2c; Table 4.3). Eosinophil levels decreased significantly ($p < 0.001$) by day 8 p.i. and remained below preinfection levels throughout the course of the infection while there was an initial insignificant ($p > 0.05$) rise in the mean proportion of the monocytes between day one and 12 p.i. (Figure 4.2d and 4.2e respectively). Thereafter, the proportions remained within preinfection levels throughout infection.

It is also noteworthy that in the two animals which selfcured, although the initial decrease in mean TWBC counts between days one and 15 p.i. was greater, the counts thereafter remained slightly below preinfection levels until day 43 p.i. when they rose. Irrespective of this, there was an apparent pattern of triphasic proportional lymphocytosis (Figure 4.2b) which was paralleled by phases of neutropaenia (Figure 4.2c). In absolute terms however, these phases did not represent overt increases or decreases in the number of lymphocytes and neutrophils (Table 4.3). As in group A, there was a steady decrease in the mean proportions of the eosinophils from day one p.i. and although there were occasional slight increases, the level remained below the preinfection means (Figure 4.2d). In

Table 4.3 **Mean absolute numbers (x 106 ml-1) of differential leucocyte counts in sheep infected with T. evansi TREU 2143. Group B selfcured.**

	Lymphocytes		Neutrophils		Eosinophils		Monocytes	
Day	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
-13	4.8	4.0	2.7	2.7	0.30	0.30	0.25	0.25
- 6	5.0	4.3	2.7	2.7	0.35	0.35	0.25	0.25
1	4.7	4.2	2.1	3.1	0.10	0.40	0.30	0.30
5	4.8	3.2	2.0	2.4	0.04	0.20	0.30	0.10
8	5.1	3.5	2.0	1.7	0.10	0.20	0.40	0.20
12	5.3	3.2	2.0	1.4	0.04	0.02	0.20	0.10
15	6.7	3.2	1.9	1.1	0.04	0.04	0.30	0.10
19	6.6	3.8	1.5	1.0	0.05	0.10	0.20	0.20
22	9.3	4.9	2.2	2.5	0.06	0.10	0.30	0.20
26	9.2	4.5	2.2	1.2	0.06	0.10	0.30	0.10
29	8.8	4.5	1.7	1.2	0.05	0.10	0.20	0.20
33	9.9	4.6	1.8	1.0	0.02	0.03	0.20	0.10
36	8.4	4.1	1.4	1.7	0.10	0.10	0.20	0.10
40	9.7	3.5	1.4	1.6	0.07	0.10	0.20	0.10
43	10.4	4.7	2.0	2.5	0.13	0.10	0.40	0.40
47	10.3	4.5	2.0	2.0	0.14	0.03	0.30	0.20
50	10.4	4.8	2.1	1.8	0.05	0.10	0.30	0.10
54	11.1	6.5	2.3	2.4	0.07	0.05	0.40	0.20
57	10.1	4.8	1.7	1.4	0.12	0.10	0.30	0.10
61	10.2	4.8	1.7	1.5	0.20	0.10	0.20	0.10
69	9.6	4.2	1.6	1.5	0.06	0.10	0.30	0.20
78	9.6	5.2	1.6	2.7	0.20	0.04	0.30	0.20
85	8.3	4.8	2.4	2.1	0.40	0.10	0.30	0.20
92	8.0	3.9	2.3	2.6	0.30	0.10	0.30	0.20
99	6.3	4.4	2.3	2.8	0.10	0.20	0.20	0.20

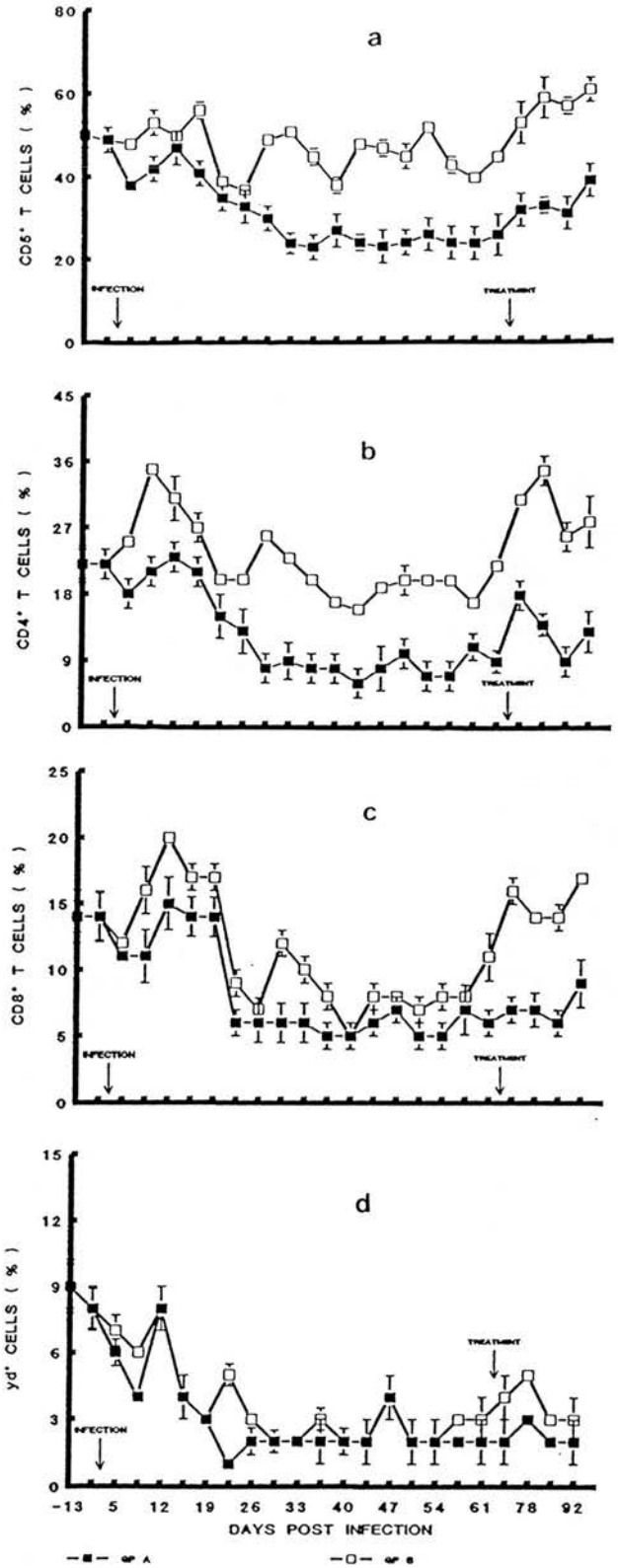


FIGURE 4.4 Sequential changes in the mean proportions of T cell phenotypes in PBLs of *T. evansi* infected sheep (Mean ± S.D.)

(a) $CD5^+$ T cells
(b) $CD4^+$ T cells
(c) $CD8^+$ T cells
(d) γδ T cells

contrast to group A, the mean proportions of the monocytes decreased below preinfection levels (Figure 4.2e).

4.3.2 Analysis of Lymphocyte Phenotypes

Infection with *T. evansi* TREU 2143 produced marked alterations in the various lymphocyte phenotypes. Representative FACS frequency histograms illustrating these changes in one animal which selfcured and one which remained infected until treated with a trypanocidal drug are shown in Figure 4.3.

4.3.2.1 Changes in T Cell Phenotypes

Graphical representations of the sequential changes in the various T cell phenotypes in the two groups are shown in Figure 4.4. In group A infection was followed by an initially significant decrease ($p < 0.05$)in the mean proportions of $CD5^+$, $CD4^+$ and $CD8^+$ cells by day 5 p.i. when compared with preinfection values, with slight recoveries which ranged variously between day 8 and 19 p.i. (Figures 4.4a, b and c respectively). Similarly, there was a significant steady decrease ($p < 0.001$; days 5, 15, 22, 40) in the mean proportion of $\gamma\delta$ T cells decreased steadily (Figure 4.4d). By day 22 p.i., the mean proportions of all T cell phenotypes in group A had shown significant ($p < 0.001$) decreases from preinfection levels, remaining so until after drug therapy. There was no significant change over time in the mean CD4:CD8 ratio in this group (Table 4.4a) despite the decreases in their proportions. This is because of a similar trend in the rate of decrease in the proportions and numbers of these cell phenotypes. The decrease in the mean proportion of $CD5^+$ cells, unlike that of other T cell subsets did not result in concurrent decrease in the mean numbers (Table 4.4b).

It is of interest to note that the mean proportions of $CD5^+$, $CD4^+$ and $CD8^+$ phenotypes of the two sheep which selfcured showed initial increases between days 8 to 19 and that thereafter those of $CD5^+$ and $CD4^+$ cells remained slightly below preinfection levels and until selfcure when they steadily increased to well above preinfection levels (Figures 4.4a,.b; 4.3a, b). In contrast, the mean proportions of $CD8^+$ cells in these two sheep following the initial slight increase, decreased well below preinfection values and remained so throughout the course of the infection (Figure 4.3c; 4.4c). Thus the CD4:CD8 ratio in the two animals showed a marked increase from days 22 to 78 (Table 4.4a) as a result of the greater decrease in the mean proportions of $CD8^+$ cells.

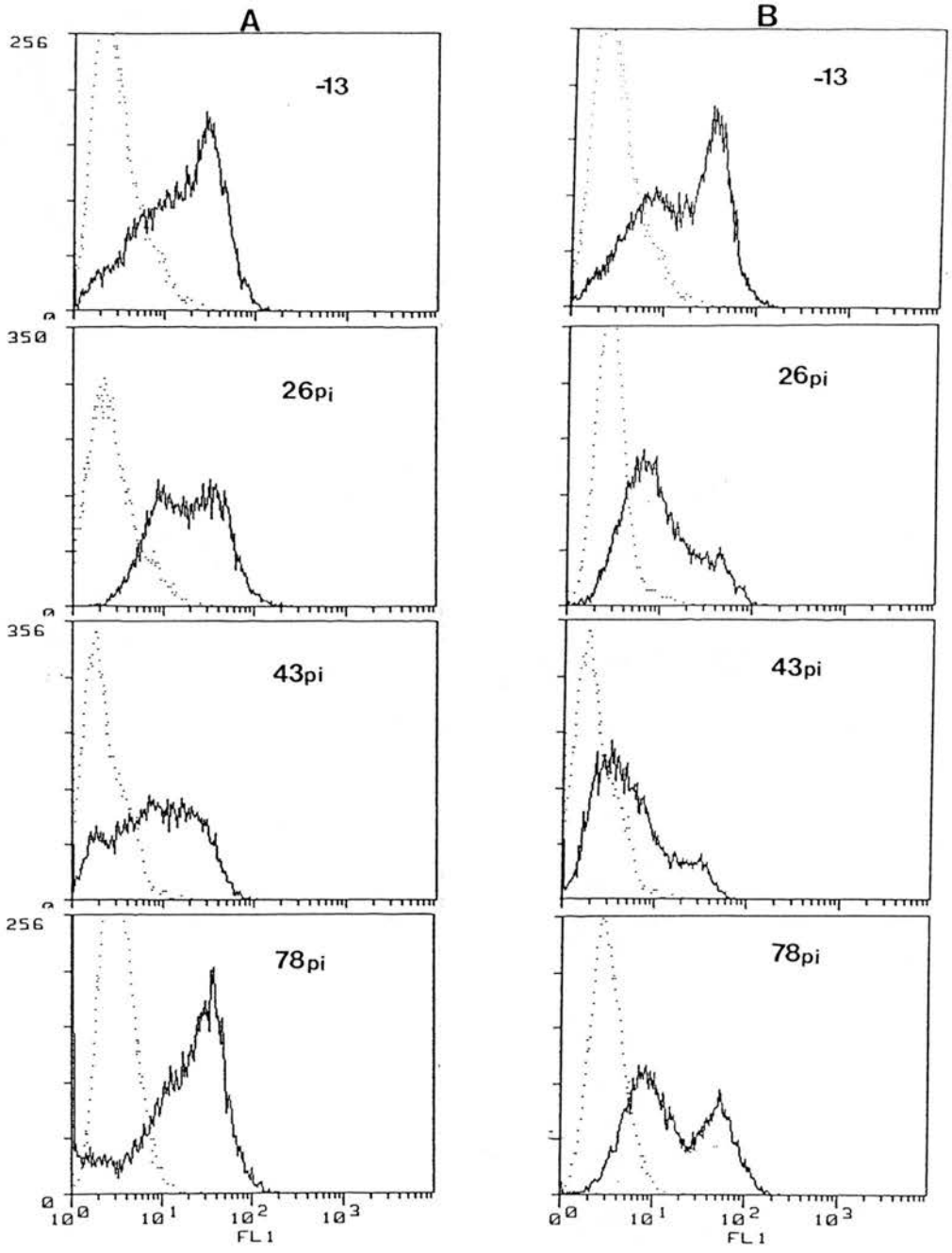


FIGURE 4.3 (a) FACS frequency histograms showing redutions in $CD5^+$ T cells in the peripheral blood of sheep infected with *T. evansi* TREU 2143. Profiles in column A are those of sheep 950 which selfcured while those in column B are those of sheep 945 which remained parasitaemic until treated. Note the marked reduction in sheep 945 and the minimal reduction in sheep 950. Day 78 is the same as two weeks after treatment for sheep 945. p.i. = days post infection.

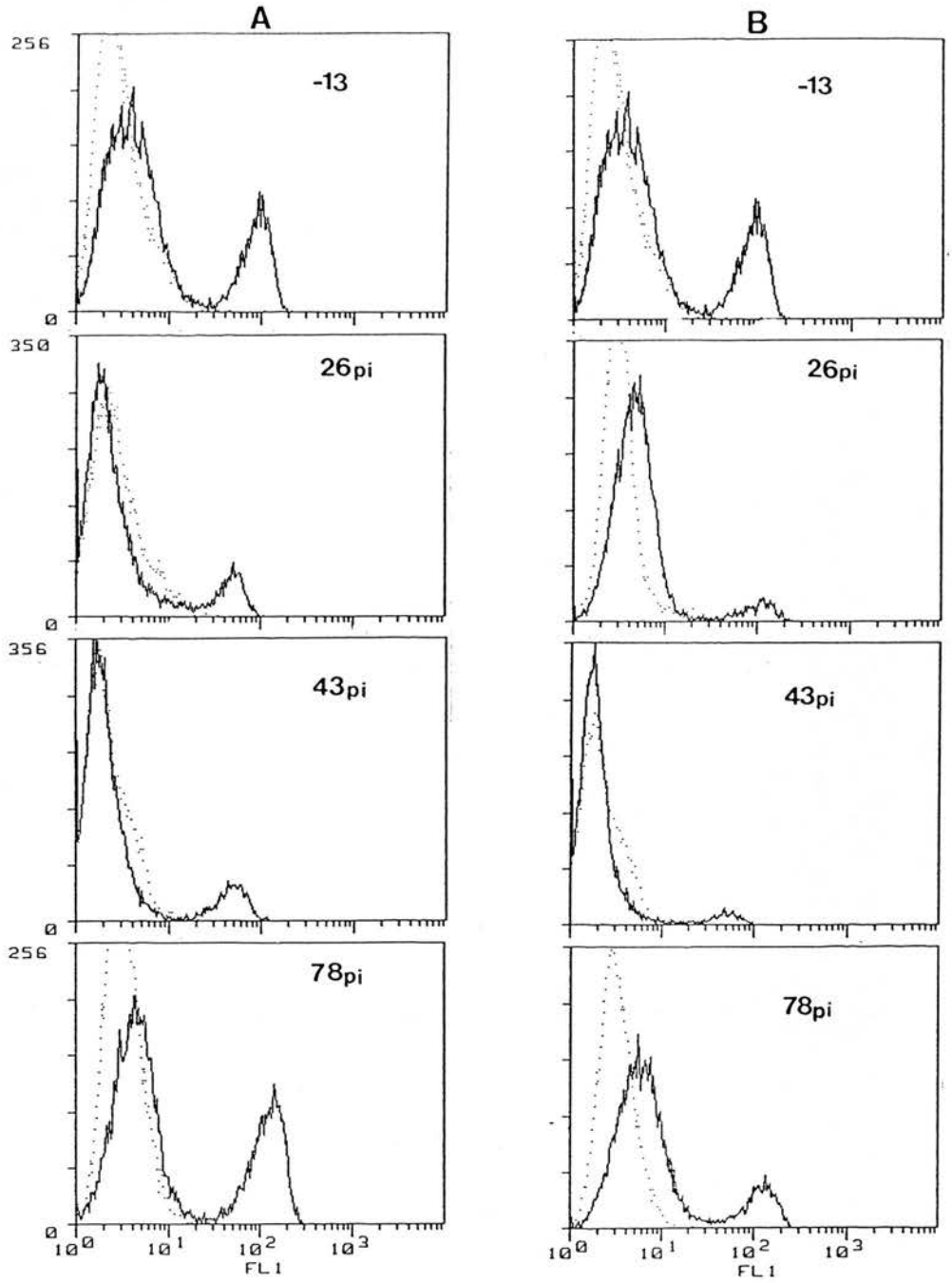


FIGURE 4.3 (b) FACS frequency histograms showing changes in PBL $CD4^+$ T cells in sheep infected with *T evansi* TREU 2143. Profiles in column A = sheep 950 (selfcured); column B = sheep 945 (parasitaemic until treated). Decreases were more marked in 945 than 950.

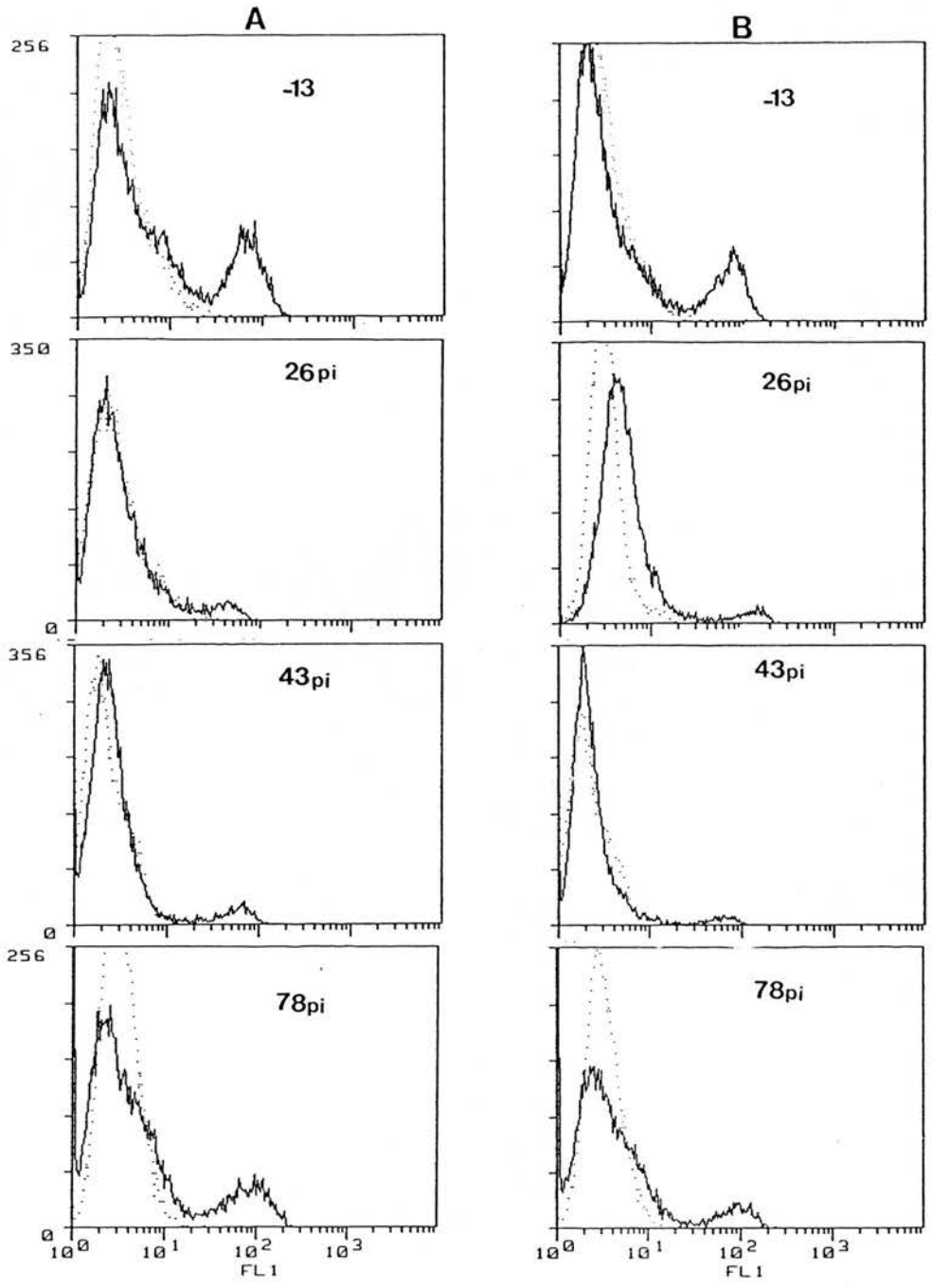


FIGURE 4.3 (c) FACS frequency histograms of changes in peripheral blood $CD8^+$ T cells in sheep infected with *T. evansi* TREU 2143. column A = sheep 950: column B = sheep 945.

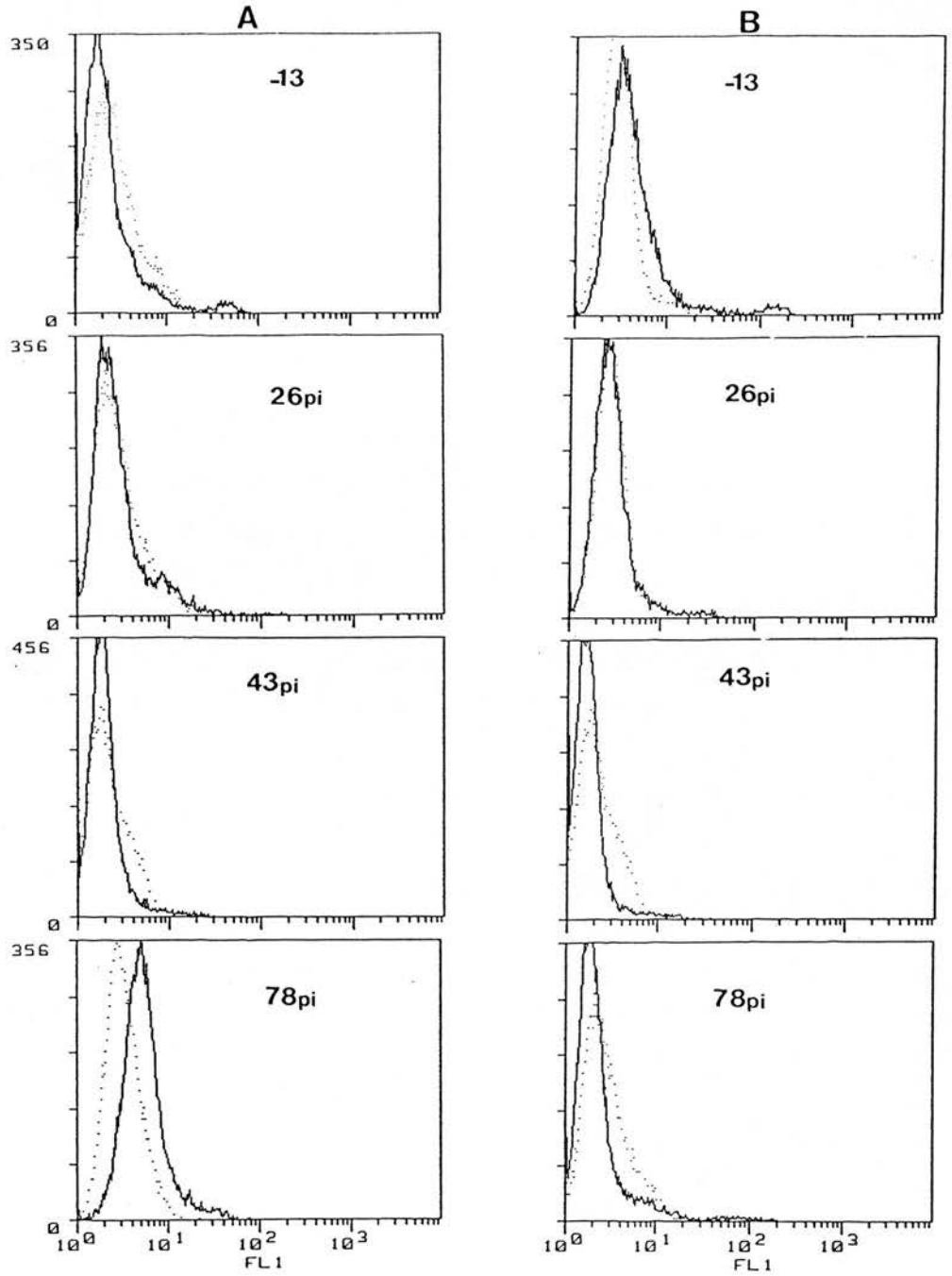


FIGURE 4.3 (d) FACS frequency histograms of peripheral blood γδ T cell changes in sheep infected with *T. evansi* TREU 2143. column A = 950; column B = 945.

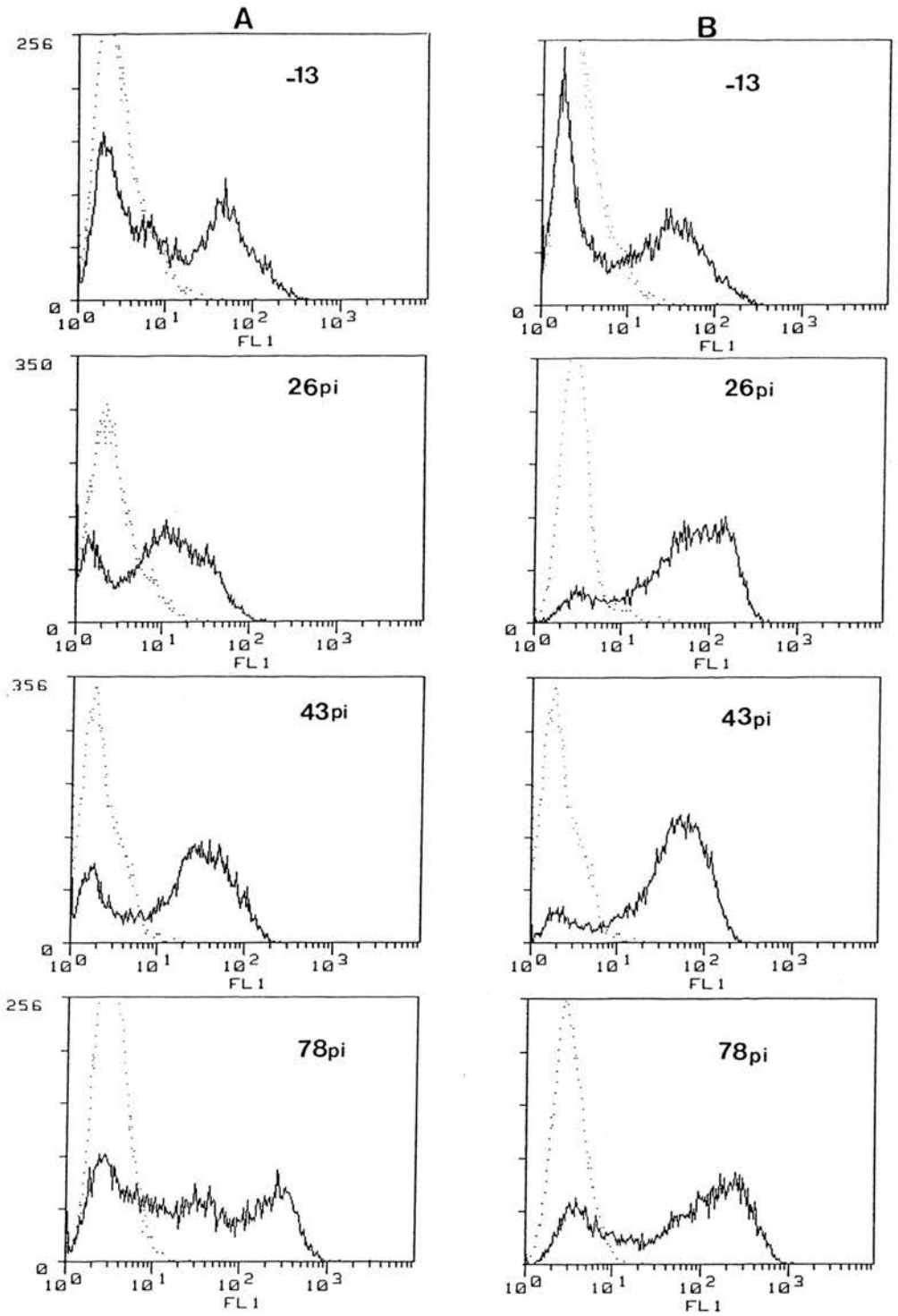


FIGURE 4.3 (e) FACS profiles showing changes in sIg^+ cells in sheep infected with *T. evansi* TREU 2143. column A = sheep 950; column B = sheep 945. Dotted lines represent negative control profiles.

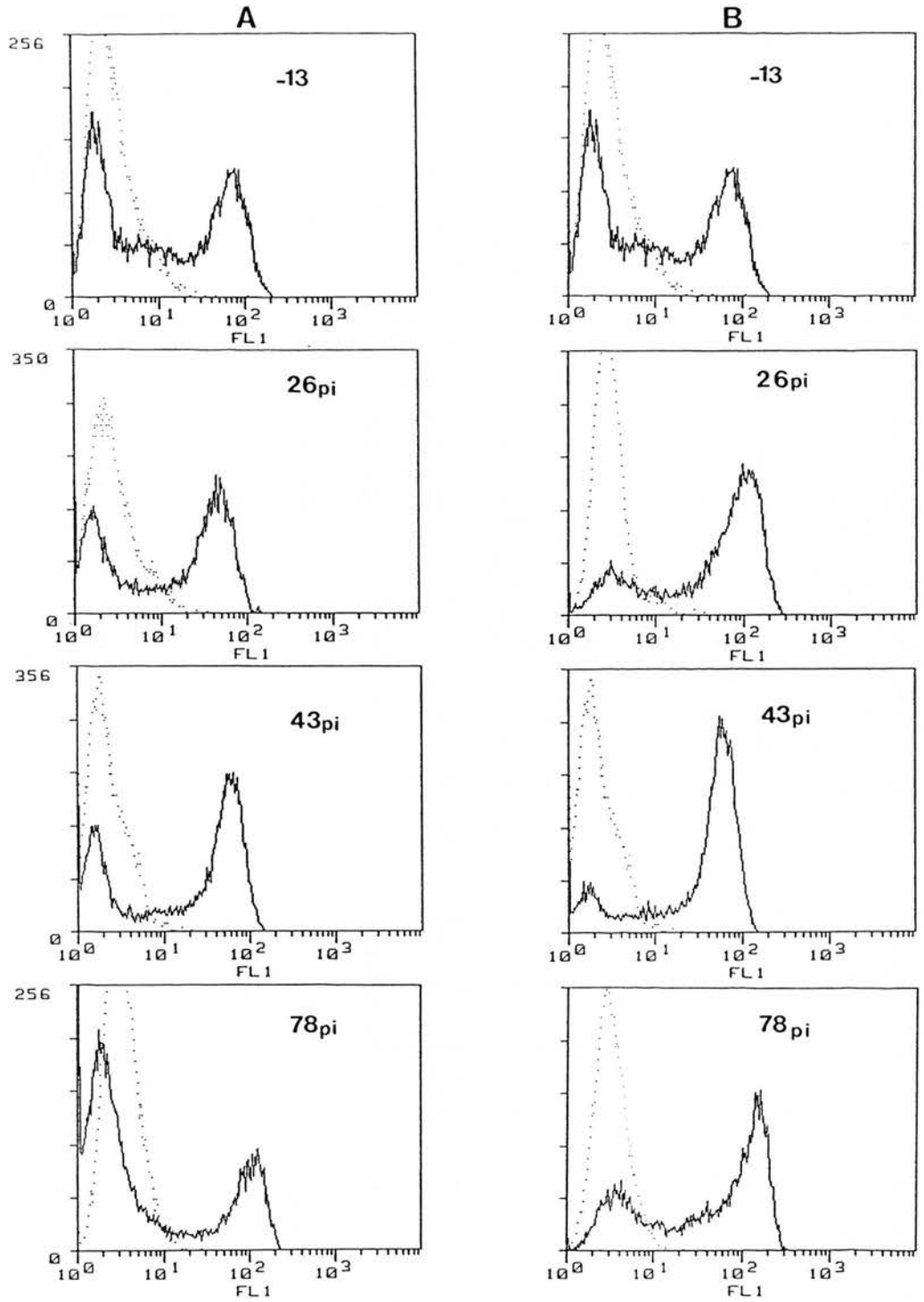


FIGURE 4.3 (f) FACS frequency histograms showing changes in CD45R$^+$ cells in sheep infected with *T. evansi* TREU 2143. A = sheep 950; B = sheep 945. Note that increases were more in sheep 945 which remained parasitaemic until treated.

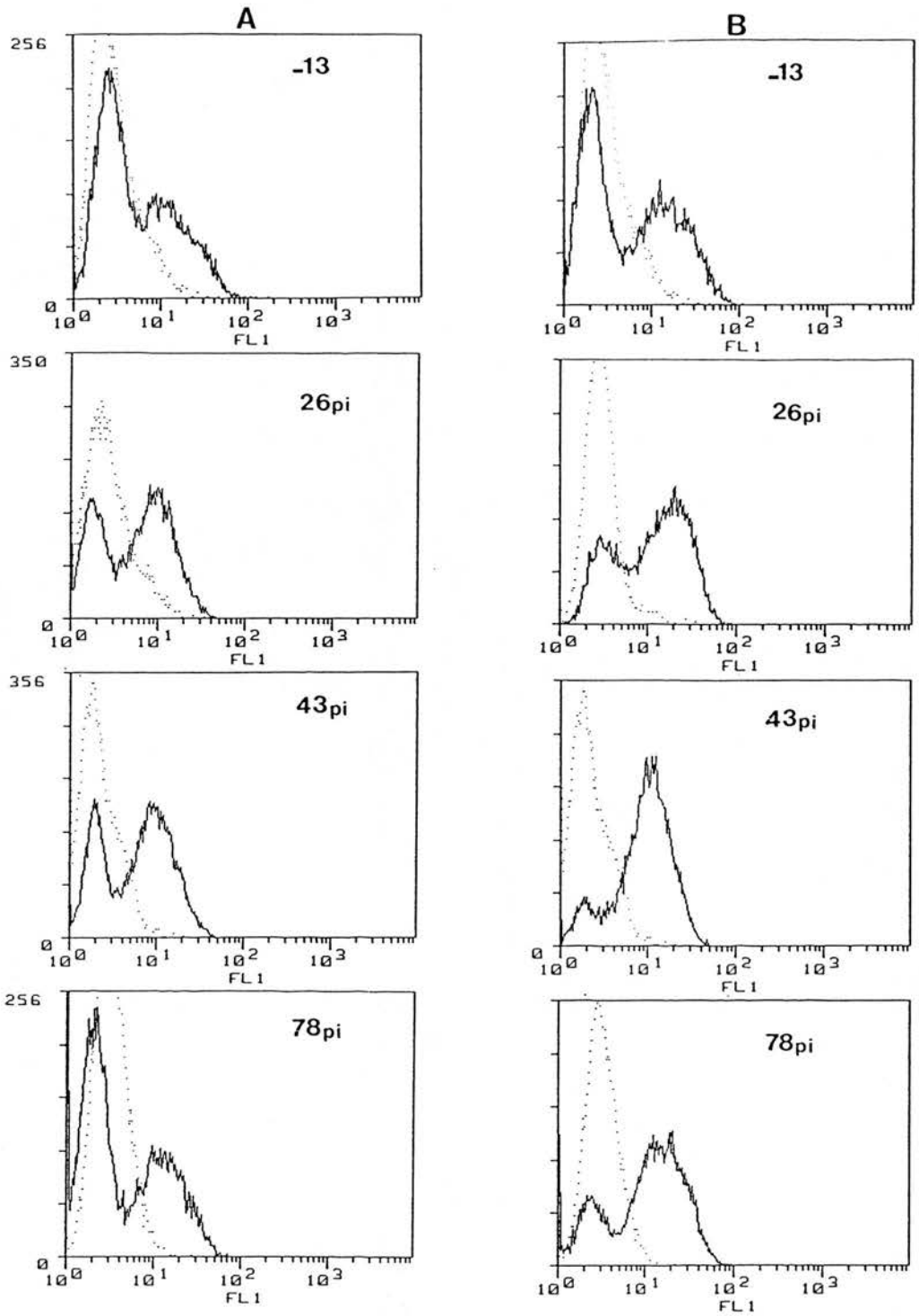


FIGURE 4.3 (g) FACS frequency histograms showing changes in $CD1^+$ cells in peripheral blood of sheep infected with *T. evansi* TREU 2143. A = sheep 950; B = sheep 945. Dotted lines represent profiles of negative control samples.

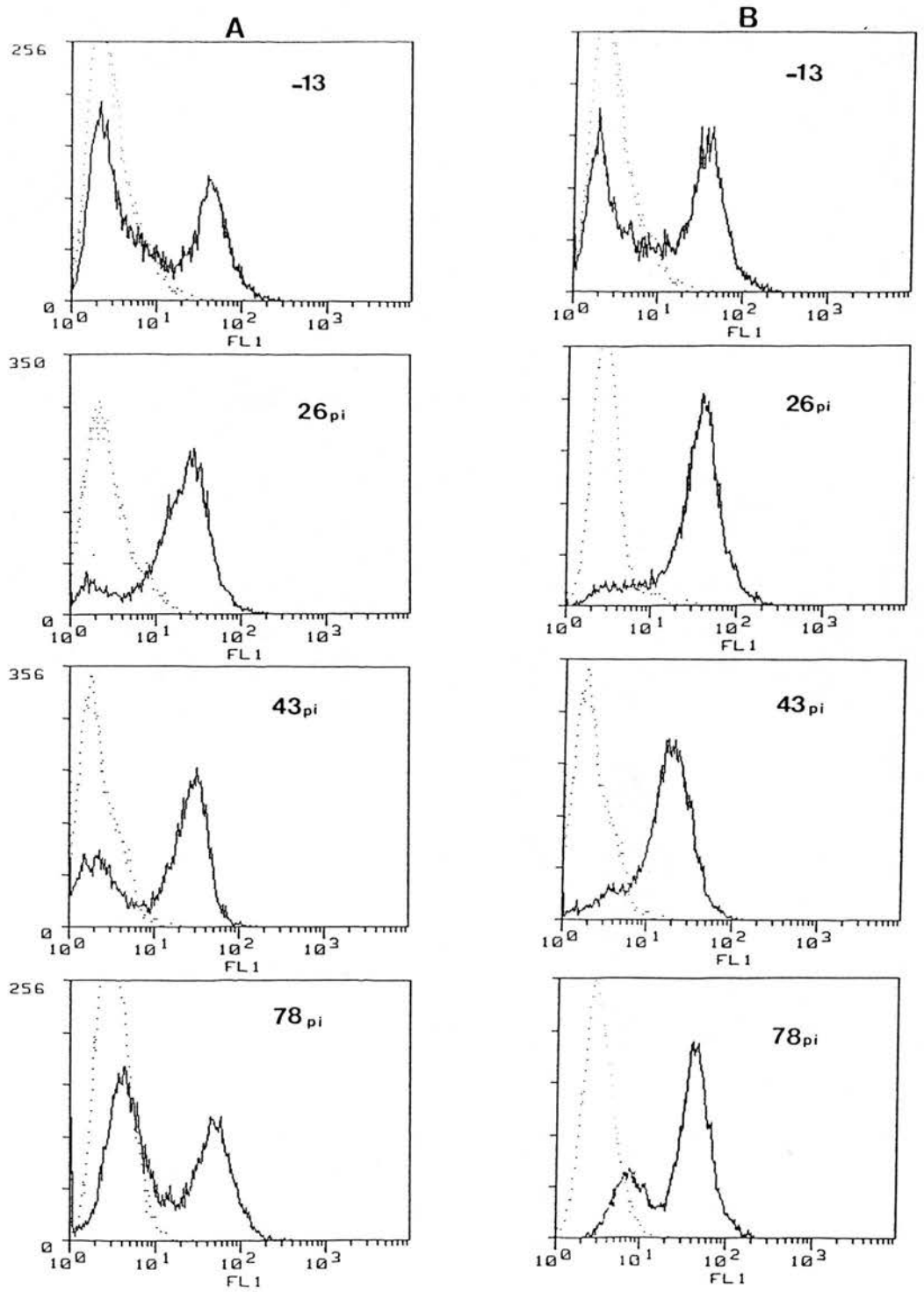


FIGURE 4.3 (h) FACS profiles showing changes in MHC Class II^+ cells in peripheral blood of sheep infected with *T. evansi* TREU 2143. A = sheep 950; B = sheep 945. Much higher increases were seen in sheep 945. Dotted lines represent prfiles of negative control samples.

Table 4.4a **The CD4:CD8 ratios in sheep infected with *T. evansi* TREU 2143. Group B animals selfcured. The ratios were calculated from the mean proportions of cells reactive with each monoclonal antibody.**

	CD4:CD8	
Day	Group A	Group B
-13	1.6	1.6
6	1.5	1.5
5	1.6	2.2
8	1.9	2.2
12	1.5	1.5
15	1.5	1.6
19	1.1	1.2
22	2.0	2.2
26	1.3	2.2
29	1.5	2.3
33	1.4	2.6
36	1.5	2.3
40	1.2	3.2
43	1.4	2.5
47	1.4	2.4
50	1.4	2.7
54	1.3	2.7
57	1.6	2.1
61	1.5	2.0
69	2.5	2.0
78	2.1	2.5
85	1.6	1.9
92	1.5	1.7

Table 4.4b **Mean absolute numbers (x 10^6 ml^{-1}) of $CD5^+$, $CD4^+$, $CD8^+$ and γδ T cells in sheep infected with *T. evansi* TREU 2143. Group B animals selfcured 50 and 54 days after the infection.**

	CD5		CD4		CD8		γδ T cell	
Day	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
-13	2.4	2.2	1.1	0.9	0.7	0.7	0.4	0.4
- 6	2.5	2.3	1.1	0.9	0.7	0.7	0.4	0.4
5	1.8	1.5	0.9	0.8	0.5	0.4	0.3	0.2
8	2.1	1.8	1.1	1.2	0.6	0.5	0.2	0.2
12	2.5	1.6	1.2	1.0	0.8	0.6	0.4	0.2
15	2.8	1.8	1.3	0.9	1.0	0.5	0.2	0.1
19	2.3	1.5	1.0	0.8	0.9	0.6	0.2	0.1
22	3.0	1.8	1.2	1.0	0.6	0.4	0.1	0.2
26	2.7	2.4	0.7	0.8	0.6	0.5	0.2	0.1
29	2.1	2.3	0.8	0.9	0.5	0.5	0.1	0.1
33	2.3	2.1	0.8	0.9	0.6	0.3	0.2	0.1
36	2.3	1.6	0.7	0.7	0.4	0.3	0.2	0.1
40	2.3	1.7	0.6	0.6	0.5	0.2	0.2	0.1
43	2.3	2.2	0.8	0.9	0.6	0.4	0.2	0.1
47	2.4	2.0	0.9	1.0	0.7	0.4	0.4	0.2
50	2.7	2.5	0.7	1.0	0.5	0.4	0.2	0.1
54	2.6	2.8	0.8	1.3	0.6	0.5	0.2	0.1
57	2.4	1.9	1.1	0.8	0.7	0.4	0.2	0.1
61	2.6	2.2	0.9	1.1	0.6	0.5	0.2	0.1
69	3.1	2.2	1.7	1.3	0.7	0.7	0.2	0.2
78	3.2	3.1	1.3	1.8	0.6	0.7	0.3	0.3
85	2.6	2.7	0.8	1.3	0.5	0.7	0.2	0.2
92	3.1	2.4	1.0	1.1	0.7	0.7	0.2	0.1

Table 4.5 **Mean absolute numbers (x 10^6 ml^{-1}) of sIg^+, $CD45R^+$, $CD1^+$ and MHC Class II^+ cells in sheep infected with *T. evansi* TREU 2143. Group B sheep selfcured 50 and 54 days after the infection.**

	sIg		CD45R		CD1		MHC II	
Day	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
-13	2.4	1.7	2.6	1.9	3.1	2.1	2.7	2.7
- 6	2.7	1.9	2.7	2.0	3.3	2.4	2.6	2.6
5	2.9	1.7	2.3	1.1	2.8	1.9	3.0	1.8
8	2.3	1.7	2.3	0.8	3.0	1.6	3.3	1.4
12	2.8	1.1	3.3	1.2	3.6	1.2	3.5	1.4
15	4.0	1.1	4.4	1.3	4.1	1.4	4.5	1.3
19	4.0	1.9	4.4	1.9	4.3	2.5	4.3	2.0
22	6.0	3.4	6.4	2.9	6.6	3.1	7.2	3.5
26	6.9	3.3	5.6	2.8	7.2	3.3	7.2	3.5
29	7.0	3.3	7.1	3.6	7.1	3.6	7.7	3.8
33	7.7	3.5	7.3	2.9	7.9	3.7	8.2	3.9
36	6.6	3.0	6.5	3.0	6.4	3.0	6.9	3.0
40	7.6	1.9	7.9	2.7	7.7	2.8	8.1	3.0
43	8.1	3.2	8.0	3.0	8.3	3.1	8.4	3.0
47	7.6	2.8	8.1	3.3	8.4	3.2	9.0	3.6
50	8.1	3.4	7.9	3.1	8.3	3.4	8.5	3.4
54	8.4	4.4	8.4	3.8	8.7	4.7	9.1	4.2
57	7.1	3.2	7.7	3.2	7.9	3.3	8.2	3.2
61	7.2	2.8	6.4	2.1	7.9	2.8	7.8	2.7
69	6.8	2.5	7.6	2.5	6.3	2.2	8.2	2.3
78	6.2	2.4	6.2	2.4	6.0	2.9	7.4	3.2
85	6.1	2.2	6.1	2.5	5.2	2.6	6.5	2.9
92	4.8	1.7	4.2	1.2	5.0	1.8	5.3	1.8

Table 4.6 **Mean numbers (x 10^6 ml^{-1}) of cells positive for MHC Class I and the leucocyte common antigen (CD45) in sheep infected with T. evansi TREU 2143. Group B sheep selfcured by day 50 and 54 postinfection.**

	MHC I		CD45	
Day	Group A	Group B	Group A	Group B
-13	4.4	4.4	4.4	4.4
- 6	4.7	4.7	4.6	4.6
5	4.8	3.1	4.6	3.0
8	5.1	3.5	4.9	3.3
12	5.3	3.2	5.2	3.1
15	6.6	3.2	6.5	3.2
19	6.4	3.8	6.5	3.7
22	8.9	4.7	8.8	4.6
26	9.2	4.5	9.0	4.3
29	8.8	4.5	8.8	4.4
33	9.9	4.6	9.7	4.5
36	8.4	4.1	8.2	4.1
40	9.5	3.5	9.5	3.5
43	10.2	4.6	10.0	4.2
47	10.3	4.5	10.3	4.4
50	10.4	4.8	10.1	4.6
54	11.1	6.4	10.8	5.5
57	9.8	4.7	9.6	4.1
61	10.0	4.7	9.6	3.3
69	9.6	4.2	9.6	4.0
78	9.6	5.2	9.4	5.0
85	8.3	4.8	8.1	4.7
92	7.8	3.9	7.8	3.9

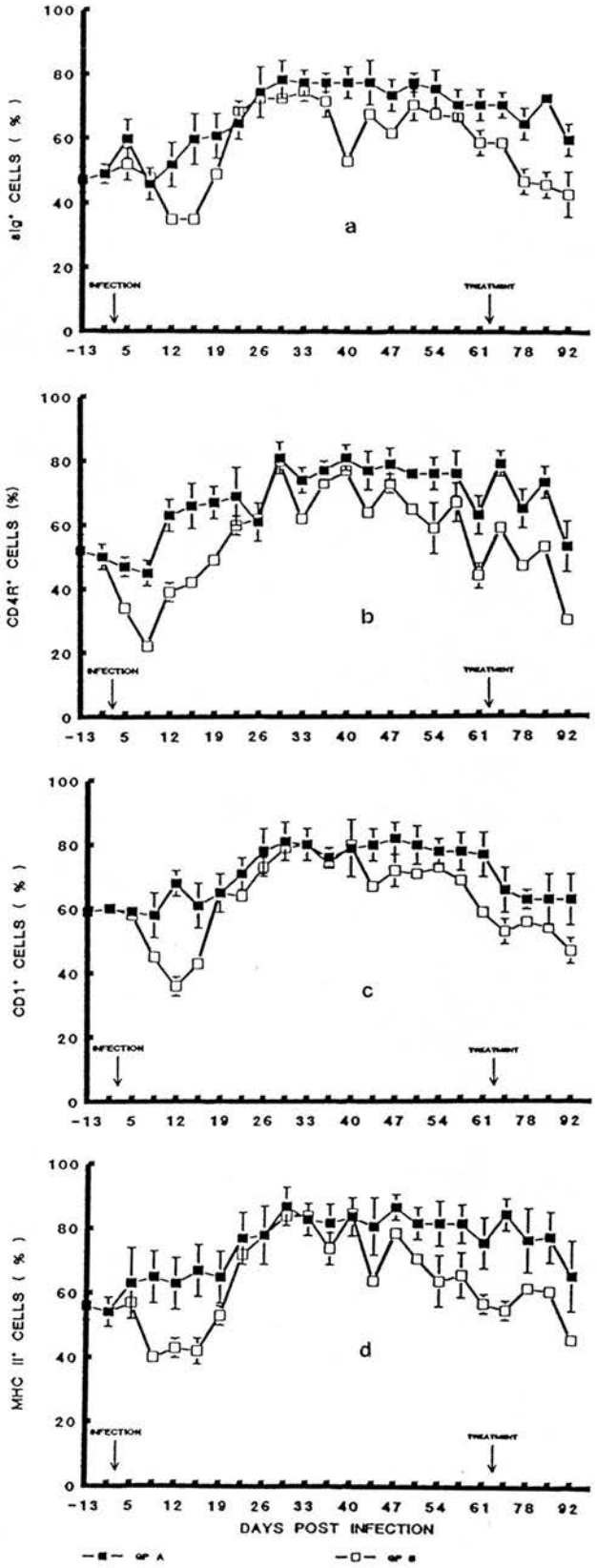


FIGURE 4.5 Sequential changes in the (Mean ± S.D.) proportions of SIg^+ $CD45R^+$, $CD1^+$ and MHC Class II^+ cells in PBLs of *T. evansi* infected sheep.

4.3.2.2 Changes in Circulating B Cells

Changes in B cell kinetics were monitored by the quantitation of sIg^+ cells using F(ab') RAS serum and by the use of mAb 20-96 (SBU-LCA p220) to determine $CD45R^+$ B cells and SBU-T6 (CD1) which stains peripheral blood B cells.

In group A, there were no significant changes ($p > 0.05$) in the mean proportions (Figure 4.5a, b and c) and numbers (Table 4.5) of sIg^+, $CD45R^+$ and $CD1^+$ cells until approximately day 22 p.i. when there were significant increases ($p < 0.01$) in the proportions of cells reactive with each of the antibodies. At this time, the mean numbers of sIg^+, $CD45R^+$ and $CD1^+$ cells had more than doubled, increasing to more than three times the preinfection levels by day 54 p.i. (Table 4.5) and remaining high even four weeks after trypanocidal drug intervention.

In group B, there was an initial decrease in the proportion of sIg^+, $CD45R^+$ and $CD1^+$ cells up to day 15 p.i. (Figure 4.5a, b and c respectively). The proportions increased gradually between days 19 to 36 for sIg^+ cells, 12 to 47 for $CD45R^+$ cells and 15 to 40 for $CD1^+$ cells. Thereafter, they gradually returned to preinfection levels and by day 61 p.i. the proportions were similar to preinfection levels. The increase in absolute numbers of cells reactive with each of the antibodies was moderate, barely reaching twice preinfection levels (Table 4.5). By day 61 p.i. they had all returned to preinfection levels.

4.3.2.3 Changes in Major Histocompatibility Complex and Leucocyte Common Antigen Expression

Changes in the number of MHC Class I^+ and $CD45^+$ cells mirrored the changes observed in total leucocyte counts (Appendix v, see also Table 4.6). The mean values for all eight sheep showed significant increases ($p < 0.001$, day 22,40) over time in both the proportions and numbers of cells expressing MHC Class II antigen (Appendix v, see also Figure 4.5d; Table 4.5).

4.3.3 Parasite-Specific Serum Antibody Responses

In the six sheep which remained persistently infected until treated,there was a 4-fold increase in serum IgM antibody levels 8 days after the infection and a steady rise was maintained throughout the infection to a 7-fold increase by day 61 p.i. (Figure 4.6). After treatment there was a gradual decline in the IgM antibody level although remaining high at 6-fold preinfection levels even 5 weeks after trypanocidal drug therapy. There was a 2-5-fold increase in the IgG_1 antibody response in group A which also showed a steady increase until day 43 p.i. It then plateaued between days 43 to 61 p.i. (Figure 4.6), rose again after treatment before beginning to decline.

On the contrary, both the IgM and IgG_1 antibody responses were apparently higher in the two sheep which selfcured. By day 8 p.i. the IgM antibody

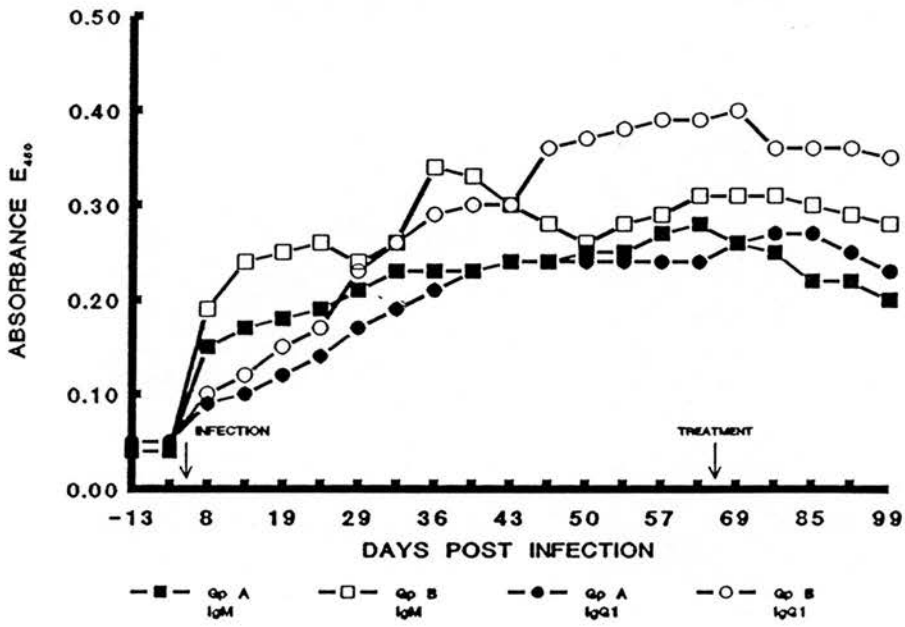


FIGURE 4.6 Parasite-specific serum antibody responses in sheep infected with *T. evansi*. Group B sheep underwent selfcure.

response had risen by 5 times preinfection levels, continuing to rise until it peaked at 7 times preinfection levels by day 26 p.i. It then dropped slightly by day 29 p.i. before rising to maximum second peak of 9-fold increase by 36 days after the infection. Thereafter, there was a steady decline to the level of the first peak of 7-fold increase by day 50 p.i. and then a third peak which plateaued at 8-fold preinfection levels between days 61 to 78 p.i. before beginning to decline again (Figure 4.6). The IgG_1 antibody response was slower, but there was a steady increase so that by 47 days after infection there was a 7-fold increase, peaking at an 8-fold increase by day 69 p.i. and then slowly declining (Figure 4.6).

4.4 Discussion

T. evansi produces a chronic infection in sheep which precipitates changes in the cellularity and dynamics of expression of various lymphocyte subsets in the blood; the degree of these changes seems to correlate with the ability of the animals to control the parasites.

There was a wide variation in the prepatent periods (p.p.p.) observed from 8 to 15 days p.i. Such variations in p.p.p. have been observed in sheep infected with *T. congolense* and were attributed to variations in the virulence of the metacyclic inocula used which were derived from different culture cocktails (Mwangi, 1991). However, since similar numbers of the same clone of *T. evansi* passaged in immunosuppressed mice were used in this study, it is possible that the variations in p.p.p. observed in this case may have arisen instead, from differences in individual animals' immune response. In fact, the p.p.p. was longer (14 and 15 days respectively) in the two sheep which eventually selfcured. Similar examples of prolonged p.p.p. have been observed in *T. congolense* infected N'dama cattle which eventually selfcured in contrast to infected Boran cattle which required drug intervention (Williams *et al.*, 1991). The parasitaemia observed in the sheep was very low and frequently cryptic. Such low parasitaemias and infrequent detection of parasites in the blood often characterise *T. evansi* infections and have been observed in rabbits (Luckins *et al.*, 1978), goats and sheep (Boid *et al.*, 1981) with natural and experimental *T. evansi* infections. Despite the low parasitaemias, there were decreases in the PCV and erythrocyte counts which were indicative of anaemia. Death in trypanosomosis is usually a result of severe anaemia and animals which are able to stem the reduction in the PCV and erythrocyte indices during the course of the infection often survive. Although the reduction in PCV and erythrocyte counts was not severe, the fact that the sheep which showed lesser decreases and even occasional increases in their PCV and erythrocyte, were able to selfcure, suggests the potential importance of the maintenance of normal PCV and erythrocyte levels in the ability of infected animals to control the infection and survive.

There was an initial reduction in the TWBC counts followed in by large

increases after the onset of parasitaemia. This sequence is in agreement with previous reports in which an early leucopaenic phase, usually corresponding to the onset of parasitaemia, is followed by persistent leucocytosis in cattle and sheep experimentally infected with *T. congolense* (Fiennes *et al.*, 1946; Naylor, 1971; Wellde *et al.*, 1974; Valli and Mills, 1980; Ellis *et al.*, 1987; Mwangi, 1991; Williams *et al.*, 1991). The leucocytosis was a result of marked lymphocytosis since both the proportions and numbers of neutrophils decreased irrespective of the increase in TWBC. Lymphocytosis is a prominent feature of rodent trypanosomosis where it is associated with polyclonal expansion of circulating B cells (Mansfield, 1978; Mansfield and Bagasra, 1978) and has also been observed in trypanosome infections in cattle and sheep (Ellis *et al.*, 1987; Mwangi, 1991; Williams *et al.*, 1991). Only moderate increases in the TWBC was observed in the two sheep which selfcured and although there were marked increases in the proportion of their lymphocytes, in absolute numbers, they represented marginal increases. Such observations were also made in sheep infected with metacyclic *T. congolense* where animals in which no increase in TWBC occurred were better able to control infection (Mwangi, 1991).

Flow cytometric analysis of peripheral blood leucocytes in animals infected with *T. congolense* showed that marked alterations occurred in the proportions and numbers of various lymphocyte subsets (Ellis *et al.*, 1987; Mwangi, 1991; Williams *et al.*, 1991). Similar changes were seen in sheep infected with *T. evansi*. Although all sheep showed significant changes in their lymphocyte subsets, there were apparent differences in the magnitude of the changes in the dynamics of the major T cell phenotypes and circulating B cells between those sheep which selfcured and those that remained persistently parasitaemic. While the proportions of $CD4^+$, $CD8^+$ and $\gamma\delta$ T cell phenotypes were reduced in in all sheep, the degree of the reduction in $CD8^+$ and $\gamma\delta$ T cells was more in the selfcured sheep, resulting in an increasing CD4:CD8 ratio. The reduction of $CD8^+$ cells and the maintenance of the level of $CD4^+$ cells in these selfcure animals may have accounted partly for the better control of parasitaemia and eventual selfcure seen in them. This is because such responses in the $CD4^+$ and $CD8^+$ subsets could subsequently enhance the helper activity of the CD4 phenotype and ensure a proper regulation of B cell proliferation and switch from IgM to IgG synthesising cells through its cytokine activity. Similar observations have been made in N'dama and Boran cattle infected with *T. congolense*. While the $CD4^+$, $CD8^+$ and $\gamma\delta$ T cell subsets were depleted in Boran cattle which had to be treated to avoid death, only the $CD8^+$ subpopulation was depleted in the N'dama, all of which eventually selfcured (Williams *et al.*, 1991). Moreover, it has been shown in rats infected with *T. brucei* that the *in vivo* depletion of $CD8^+$ cells using specific anti-CD8 mAb resulted in the abrogation of IFN-γ production, suppression of parasite growth and prolonged survival of the rats (Bakhiet *et al.*, 1990). The T_H2 subset of $CD4^+$ T cells, which is a potent B cell

activator and provider of antigen-specific help to B cells, is strongly inhibited by IFN-γ (Mosmann and Moore, 1991). IFN-γ is a product of $CD8^+$ cells and the T_H1 subset of $CD4^+$ cells. Hypothetically, it is possible that in these selfcure sheep, the reduction in $CD8^+$ cells enhanced the T_H2 action which in turn may have potentiated its provision of specific help to B cells by producing IL-10. IL-10 actively inhibits cytokine release by T_H1 (Mosmann and Moore, 1991).

The decrease in the proportions of the $CD5^+$ cells did not result in a decrease in the absolute numbers of this subpopulation. A number of reasons could possibly account for this. One simple explanation is that the drop in the proportions of $CD5^+$ cells is merely a reflection of the large increase in the proportion and numbers of B cells. However, the absolute numbers of $CD4^+$, $CD8^+$ and γδ T cells decreased, though to a relatively lesser extent in comparison to the decreases observed in their respective proportions. Nevertheless, reductions in these three phenotypes no matter how minor, should ideally be reflected in the numbers of $CD5^+$ cells since they are all positive for the CD5 antigen. Hence, although it was not investigated, it is tempting to speculate that the absolute numbers of $CD5^+$ cells may have remained unchanged because they had possibly been added to by increased numbers of the B cell population expressing the CD5 antigen after the infection. This speculation derives support from recent reports that there is a considerable increase in the proportion and numbers of $CD5^+$ B cells in cattle infected with *T. congolense* (Williams *et al.*, 1991).

Analysis of the sIg^+, $CD45R^+$ and $CD1^+$ cells revealed significant increases in the dynamics of B cells in all the sheep. While there was more than 3-fold increase of B cells in the sheep which remained persistently infected until treated, the increase in the two that selfcured was only 2-fold. Moreover, the increase in the latter sheep was biphasic with the second peak coinciding with the period of parasite control and selfcure. Increases in circulating B cells are a prominent feature of trypanosome infections (Labohm, 1982). In cattle and sheep infected with *T. congolense*, large increases in circulating B cells were reported as the infection progressed (Mwangi, 1991; Williams *et al.*, 1991). However, Ellis *et al.* (1987) were unable to demonstrate such increases in circulating B cells in cattle infected with *T. congolense*, although this may have been a result of the short duration of their experiment, which lasted for only 21 days. In the present work, significant increases in circulating B cells commenced by approximately day 22 p.i. and this finding corroborates the work of Mwangi (1991) and Williams *et al.* (1991) in which increases were observed to commence on days 25 and 21 p.i. respectively.

It is interesting that the findings by Mwangi (1991) in sheep, were similar to those of Williams *et al.* (1991) in cattle similarly infected with *T. congolense* and even more important that the observations made with *T. evansi* in sheep in this study parallel both their findings. The significance of this is that sheep which are

easily available and easier to handle, can therefore serve as an appropriate experimental model for the study of various trypanosome species despite criticisms that they are better studied in their various natural hosts.

Although the presence of higher levels of circulating B cells has been suggested as a possible reason for the ability of N'dama cattle to control *T. congolense* infection (Ellis *et al.*, 1987), the results of this study suggest that this may not depend simply on increasing B cell numbers *per se*, but probably on the rate and pattern of B cell proliferation and its interaction with the pattern of $CD4^+$ and $CD8^+$ T cell responses in relation to the isotype specificity and affinity of the antibodies produced.

Increases in the proportion and numbers of cells expressing the MHC Class II antigen in all sheep showed similar trends to those of the circulating B cells. High levels of MHC Class II antigen expression has been reported in cattle and sheep infected with *T. congolense* (Ellis *et al.*, 1987; Mwangi *et al.*, 1990; 1991; Mwangi, 1991). Such increases in MHC Class II expression may be a reflection of the increases in circulating B cells which express the Class II antigens constitutively (Singer and Hodes, 1983; Hopkins *et al.*, 1986; Lalor *et al.*, 1986; Emery *et al.*, 1987). However, MHC Class II antigen is also expressed by activated T cells (Singer and Hodes, 1983) and can thus also be an important indicator of the state of activation of T cells and therefore of utility in assessing the T cell responses to parasites (Ellis *et al.*, 1987).

The microELISA results for *T. evansi*-specific antibody responses showed that both IgM and IgG_1 antibodies were produced during the course of the infection. This is generally similar to reports from both naturally and experimentally induced trypanosome infections. For example, natural or experimental infection of cattle with *T. brucei*, *T. congolense* or *T. vivax* resulted in increases in the serum IgM and IgG antibody levels of up to 2-10 times and 2 times preinfection levels respectively (Luckins, 1972; 1976). Similarly, in sheep naturally infected with *T. congolense* alone or with a mixture of *T. congolense* and *T. vivax*, and in those experimentally infected with *T. brucei* or *T. congolense* (Mackenzie, Boyt and Nesham, 1979), there were significant increases in both the IgM and IgG antibody levels during the course of the infection.

However, it might be necessary to point out that whereas these similarities occur, there are fundamental differences between the present study and those of the aforecited authors. Whilst this study was concerned with parasite-specific antibody responses, the earlier authors assayed pathological increases in serum antibody responses without selection for their parasite specificity. More significant is that the findings of this study are very similar to those reported in experimental infection of cattle and goats with clones of *T. vivax* which resulted in high titres of parasite-specific serum IgM and IgG_1 antibodies with the IgG_1 antibody response appearing

at levels comparable with IgM antibody 4 days later than the IgM response (Vos and Gardiner, 1990). However, the increase in serum IgG_1 observed in our study, differed from those observed in Zebu cattle infected with *T. congolense* and *T. vivax* in which there was little change in the serum IgG_1 and IgG_2 antibody levels for up to 130 days post infection (Luckins, 1976a). It also differed from those of Houba *et al.* (1969) who found no increases in IgG antibody levels in monkeys infected with *T. brucei*, *T. gambiense* and *T. rhodesiense*. Again these differences may be as a result of the fact that the authors did not assay for parasite specificity of the IgG isotypes.

Although there were increases in the serum IgM and IgG_1 antibody levels in all sheep in this study, there was a definite difference in the pattern of responses in the two animals which selfcured and those which remained parasitaemic until treated. The latter animals showed a steady rise in IgM antibody level until treatment when there was a slow decline, whereas IgG_1 rose until it plateaued between days 43-61, rising again above the IgM level only after treatment. On the other hand, in the former sheep, there were three definite peaks of IgM antibody response and two of IgG_1 response with the second peak in both cases exceeding the first. Moreover, while the IgG_1 levels showed further increases by day 47 p.i., those of IgM declined, remaining so until the end of the experiment. Noteworthy is the fact that this further increase in the level of IgG_1 isotype response when the IgM level was declining, coincided with the onset of selfcure and it is tempting to infer that their ability to eliminate the parasites was a result of this later increase in IgG_1 response. In the past, drug intervention has hindered observation of changes in Ig isotypes associated with selfcure in trypanosome-infected animals. This is the first report in which it has been possible to compare the changes in lymphocyte subsets and Ig isotype responses associated with selfcure. However, the ability of wildebeast and Zebu cattle to control infection with *T. brucei* has been linked to a classical sequence of antibody production and isotype switch from initial high levels of IgM antibody to subsequent IgG_1 and IgG_2 antibodies (reviewed by Mulla and Rickman, 1988). The secondary increase in IgG_1 levels in the six sheep which remained parasitaemic occurred only after drug treatment. This may be as a result of the release of common antigens from the killed and disintegrating trypanosomes to which the animals had been sensitised. On the other hand, it could be that the clearance of the trypanosomes by trypanocidal therapy effectively removed the pathological barrier to Ig isotype switching, thus enabling a secondary IgG_1 response at a time when IgM level was declining.

It is significant to note that while the pattern of Ig response differed in these sheep, the two selfcure animals at all time points, showed apparently higher levels of both IgM and IgG_1 antibodies than the rest, which suggests depressed or reduced parasite-specific antibody response in the persistently parasitaemic sheep. Such reduction in the level of parasite-specific antibody response has been observed

in rodents infected with *T. brucei* (Hudson and Terry, 1979; Sacks and Askonas, 1980) and in cattle infected with *T. brucei* or *T. congolense* (Nantulya *et al.*, 1982; Morrison *et al.*, 1985). Thus while there was apparent persistent hypergammaglobulinaemia of IgM and IgG_1 in these persistently parasitaemic group, it was not enough to effect parasite clearance. In the circumstances, such differences can only be attributed to the observed differences in the levels and patterns of expression of $CD4^+$ and $CD8^+$ T cells and on the expansion of the circulating B cell population. It is paradoxical that while there was significant increases in the proportions and numbers of circulating B cells in the persistently parasitaemic animals, their IgM levels still remained below those of the selfcure sheep. The rapid and uncontrolled expansion of B cells has often been used to explain the superabundance of IgM in trypanosome infections (Terry *et al.*, 1973; Urquhart *et al.*, 1973; Murray *et al.*, 1974a) and the presence of heterophile antibodies (Houba *et al.*, 1969; Klein *et al.*, 1970; Boreham and Facer, 1974; Mackenzie and Boreham, 1974). Results of this study, apparently argue against B cell expansion as the main reason for high IgM levels in trypanosome infections since IgM levels were higher in the selfcure sheep which had smaller increases in B cells than the rest of the sheep. Note however, that without the accident of having animals which selfcured and thus the benefit of observing the changes associated with selfcure, the findings in all sheep would simply have corroborated previous reports as that of sustained and increasing IgM responses associated with large increases in circulating B cells. In any case, it may be worth mentioning again that this study assayed IgM responses against the specific clone of *T. evansi* which elicited it, a fact which is not true for the majority of the previous reports of IgM hypergammaglobulinaemia during trypanosomosis.

In conclusion, this study has shown that *T. evansi* infection significantly alters the dynamics of expression of the major effector lymphocyte subsets in the peripheral blood which appeared to be associated with the level and trend of antibody responses to infection. Sheep which maintained normal levels of CD4 expression concurrently with reduced CD8 expression by T cells and had moderate B cell increases produced sterilising levels of antibody and were able to apparently switch from IgM to IgG_1 production. On the other hand, in sheep in which concurrent reduction in $CD4^+$ and $CD8^+$ T cell subsets was accompanied by marked increases in circulating B cells, lower parasite-specific antibody level was observed. Moreover, the animals failed to eliminate the parasites and it seemed that Ig isotype switch did not occur until after trypanocidal drug treatment.

It is therefore opined that although T cell subsets play no direct role in trypanosome killing, it is likely that alterations in their demographics by an ongoing infection affects the level and pattern of antibody responses and therefore, the eventual outcome of trypanosome infection as a disease. Interesting as the findings

in this experiments may be, speculation on the phenomenon of selfcure during trypanosomosis is based on data drawn from only two animals in which the process occurred. It will therefore be premature to attach too much significance to or draw far reaching conclusions from the observations so far made. However, the sheep appears to be a good experimental model for the study of the immunopathology of pathogenic trypanosomes and further study of the subject in a much larger population of sheep will greatly enhance the validity or otherwise of the conclusions drawn from this study.

This notwithstanding, the effect of *T. evansi* infection on the various parameters measured in this study was subjected to statistical analysis using the Student's t-test. Paired t-test of the preinfection values of all parameters against those at various time points after the infection showed significant differences between pre- and post-infection values. Details of the analysis of the pooled data for all the sheep are presented in appendix five.

CHAPTER FIVE

Suppression of *Pasteurella*-Specific Immune Responses and Increased $CD5^+$ B Cell Expression

5.1 Introduction

In *T. evansi* infection in sheep there are decreases in the relative proportions of $CD5^+$, $CD4^+$, $CD8^+$ and γδ T cell phenotypes but increases in the proportions and numbers of circulating B cells. In addition, it emerged that the degree of these changes especially in the relative proportions of $CD4^+$ and $CD8^+$ cells appeared to affect the pattern and level of parasite-specific IgM and IgG antibody responses and the ability to control the infection. For instance, in infected sheep which eventually selfcured, there was little reduction in the proportion of $CD4^+$ cells while that of $CD8^+$ cells was considerably reduced (Chapter 4). Moreover, although there were increases in B cell proportions and numbers, they were higher in the animals which failed to selfcure.

The implication of this is that increases in circulating B cells during trypanosomosis do not *per se* result in the production of higher levels of parasite-specific antibodies capable of controlling the infection. This has remained the paradox of the immunopathology of trypanosome infections in general. However, in *T. congolense* infection in cattle, similar increases occur in circulating B cells and it has recently been shown that up to 90 percent of such B cells express the CD5 antigen (Williams *et al.*, 1991). Such B cell populations in human and mouse are known to produce antibodies which are auto- and polyreactive in action and bind antigen with low affinity (Hayakawa *et al.*, 1983; 1984; Casali and Notkins, 1989). If, as in the case of *T. congolense* infection in cattle, *T. evansi* also induces expansion of $CD5^+$ B cell population in infected sheep, then it might explain why these sheep failed to produce sterilising antibodies despite the increased numbers of B cells.

It is possible that the suppression of immune responses against heterologous antigens during the course of trypanosome infection in animals may also be related to these alterations in the proportions of various effector lymphocyte phenotypes. Such relationships have been established in various diseases of animals and man. For instance, infection of sheep with bovine respiratory syncytial virus (BRSV) results in marked decreases in the proportion of $CD5^+$ and $CD4^+$ T lymphocytes and is characterised by non-specific immunosuppression resulting in increased susceptibility to secondary *Pasteurella haemolytica* infection (Sharma and Woldehiwet, 1990; 1991b; Sharma *et al.*, 1990). In humans, the increased susceptibility to secondary infections seen in AIDS patients results in part from the selective depletion and expansion of the $CD4^+$ and $CD8^+$ T cells respectively (Giorgi and Detels, 1989). Moreover, activation of the $CD8^+$ T cell phenotype (putative T suppressor cells) is implicated in the specific suppression of immune responses to *Plasmodium falciparum* antigens during acute malaria (Riley *et al.*, 1988; 1989).

In the following experiments, the effects of *T. evansi* infection on the local

skin reaction and systemic humoral immune responses of sheep to *Pasteurella haemolytica* vaccine were studied. The ovine B cell population was also analysed by two-colour indirect immunofluorescence technique and flow cytometry in order to establish whether infection with *T. evansi* induces increased expression of the CD5 antigen on B cells. In addition, the effects of the infection on the dynamics of expression of the major T cell subsets and the MHC Class II antigen following the administration of *Pasteurella* vaccine were studied.

5.2 Materials and Methods

5.2.1 Experimental Animals, Trypanosomes and Infection of Sheep

Suffolk sheep of either sex aged 9 months were purchased from Moredun Research Institute, Edinburgh and used in this study. None of the animals had been vaccinated previously against pneumonic pasteurellosis. They were housed and fed as described in section 3.1. Three of the sheep were infected i.v. with 2×10^6 bloodstream trypomastigotes of *T. evansi* TREU 2143 as shown in Table 5.1.

5.2.2 *Pasteurella* vaccine and vaccination procedure

The *Pasteurella haemolytica* vaccine used in this study, the dosage and the route of administration have been described in Section 3.8.1. The vaccination schedule is as shown in Table 5.1.

Table 5.1 **Experimental Design**

Sheep	Infection status	Days after infection when vaccine was administered		Days of trypanocidal drug treatment
		Primary dose	Booster dose	
322*	TREU 2143	28	57	*
445	"	28	57	90
479	"	28	57	90
316	Control	**	**	-
522	"	**	**	-

* Selfcured, no drug treatment; ** Vaccinated simultaneously with the infected sheep

5.2.3 Sampling Methods

Blood was collected for serum preparation and isolation of peripheral blood leucocytes. Samples were obtained once weekly for two weeks prior to infection and/or vaccination, twice weekly during the week immediately after the primary and secondary vaccination and once weekly at other times. PBLs for two-

colour indirect immunofluorescence were isolated by tris-ammonium chloride lysis as described in Section 3.5.1. Serum for the assay of *Pasteurella*-specific antibodies was prepared as described in Section 3.8.1. Cytospin smears and thin blood films for the study of granulocyte responses following infection and vaccination were prepared as described in Sections 3.5.3 and 3.5.4 respectively.

5.2.4 Local Responses at the Site of Vaccine Administration

Local inflammatory responses at the site of *Pasteurella* vaccine administration were assessed visually, by palpation and by the measurement of skin thickness at the vaccination site. These assessments were facilitated by cleanly shaving the area of vaccine administration. The skin thickness at the site of inoculation was measured daily with a pair of vernier calipers for up to 30 days after the primary and secondary vaccine administration respectively.

5.2.5 Two-colour Immunofluorescence Staining

The monoclonal antibodies SBU-T1 and VPM 30 (Table 3.1a) were used for the two-colour indirect immunofluorescence staining to assess the responses of peripheral B cell and $CD5^+$ T cell subsets to *Pasteurella* vaccine administration and how prior infection with *T. evansi* TREU 2143 affected them. SBU-T4, SBU-T8, SBU-T19 and SBU-II were used to assess the effect of infection on CD4, CD8, $\gamma\delta$ T cell subsets and MHC Class II responses respectively, to *Pasteurella* vaccine administration. The staining techniques used are fully described in Section 3.6.3.2 and the method of acquisition and analysis of cell data in Section 3.6.5.

5.2.6 Immunoassay of *Pasteurella*-Specific Antibodies

Total antibody response as well as isotypic (IgG_1, IgG_2, IgM) serum Ig responses to *Pasteurella* vaccination were assayed using indirect and double-sandwich microELISA techniques respectively. Results were quantified photometrically and recorded as optical densities (O.D).

5.3 Results

5.3.1 Parasite Kinetics

Parasitaemia was characteristically scanty, the trypanosomes first being detected by HCT on day 9 p.i. in sheep 445 and on day 13 p.i. in sheep 322 and 479. Parasitaemia peaked in all sheep on day 23 p.i. when an average of one parasite/16 microscope fields was detected in wet blood film preparations. Sheep 322 ceased to show detectable parasitaemia by day 48 p.i. and mice inoculated with its blood on day 52 p.i. failed to become infected within 30 days observation. Hence,

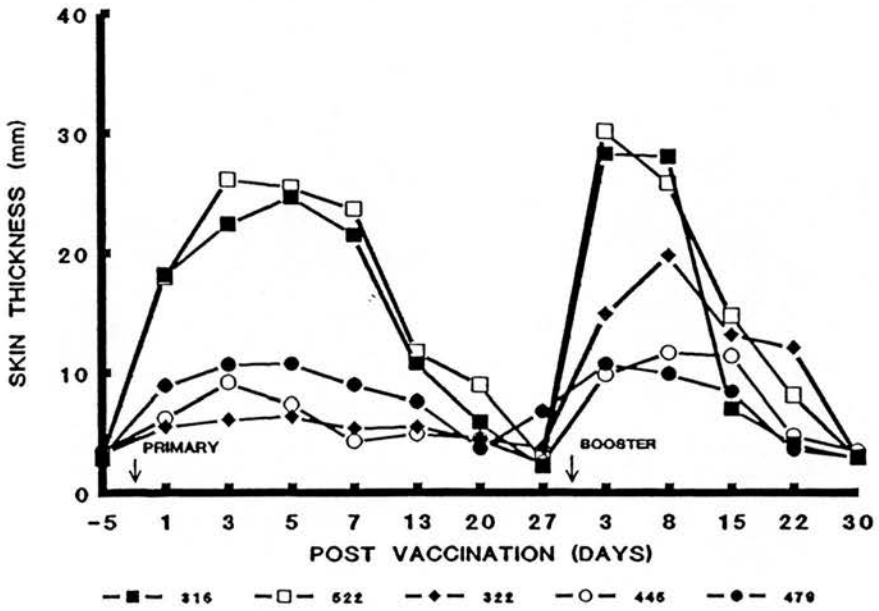


FIGURE 5.1 Skin thickness after *Pasteurella* vaccination in *T. evansi* infected and uninfected sheep.

Sheep 316 and 522	=	Uninfected/vaccinated
Sheep 445 and 479	=	Infected/vaccinated/drug treated
Sheep 322	=	Infected/vaccinated/selfcured

it was concluded that this sheep had undergone selfcure 48 days after infection. Sheep 445 and 479 remained parasitaemic until after they were treated on day 90 p.i. with 5 mg kg^{-1} of Cymelarsan (Mel Cy). Mouse inoculation with blood collected from these sheep 7 days after treatment showed that the single injection of Mel Cy was sufficient to clear the parasites.

5.3.2 Responses to *Pasteurella* Vaccine Administration

5.3.2.1 Local Primary Responses

Primary inoculation of control sheep 316 and 522 with *Pasteurella haemolytica* vaccine elicited classical local inflammatory responses represented by swelling, erythema, warmth and tenderness upon touch within the first three days of vaccination. However, in sheep 322, 445 and 479 all of which had been infected with *T. evansi* TREU 2143 28 days previously, this response was largely suppressed. The active inflammatory response was also accompanied, 3 days after vaccination, by a painless induration and thickening of the skin at the site of vaccination. In the uninfected sheep there was up to an 8-fold increase in the skin thickness compared with only a 3-fold increase in the infected animals (Figure 5.1). This painless skin induration generally abated by day 14 p.v. and by day 23 p.v. the skin had returned to prevaccination thickness. There was no tissue destruction and sloughing-off at the site of vaccination.

5.3.2.2 Local Secondary Responses

Following secondary (booster dose) administration of *Pasteurella haemolytica* vaccine, the inflammatory response was not as acute as in the primary response. In the uninfected sheep, the erythematous skin induration appeared in the form of a plaque reminiscent of a delayed type hypersensitivity reaction two days post booster dose (p.b.) administration, peaked between days 3 to 8 p.b. and by day 19 p.b. had receeded to pre-vaccination levels (Figure 5.1). This secondary response was anamnestic in that there was up to an 11-fold increase in skin thickness from pre-vaccination level of 3 mm to 35.6 mm on day 5 p.b. in sheep 522. In the infected animals, sheep 445 and 479 showed no differences in the thickness in response to primary and secondary vaccine administration (Figure 5.1). In contrast, sheep 322 which subsequently selfcured responded anamnestically as the uninfected controls, with up to a 7-fold increase in its skin thickness following secondary vaccine administration (Figure 5.1).

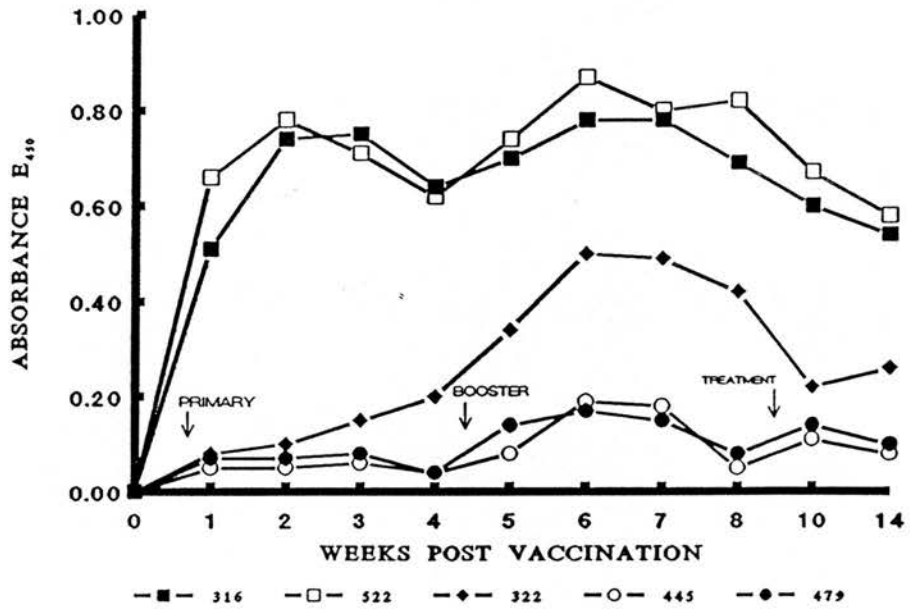


FIGURE 5.2 Serum antibody responses to *Pasteurella* vaccine in *T. evansi* infected and uninfected sheep.

Sheep 316 and 522	=	Uninfected/vaccinated
Sheep 445 and 479	=	Infected/vaccinated/drug cured
Sheep 322	=	Infected/vaccinated/selfcured

5.3.2.3 Systemic Leucocytic Responses

Plates 5.2a-d are representative photographs of point cytospin preparations of sheep PBLs following primary *Pasteurella* vaccine administration. Primary vaccination was accompanied by early systemic mobilisation of neutrophils in both control sheep (Plate 5.1a). This response was absent in the infected animals (Plate 5.1b) but was replaced by persistent lymphocytic leucocytosis orchestrated by the trypanosome infection. Seven days after vaccination, the neutrophilia of the control animals had receeded and was replaced by a mixture of lymphoblasts, small lymphocytes and neutrophils (Plate 5.2c). In contrast, in the infected sheep, there was continuing lymphocytic leucocytosis, comprising mainly blastoid cells (Plate 5.1d).

Following booster vaccination, the response in the control animals was characterised largely by the presence of both lymphocytes and neutrophils. Sheep 445 and 479 on the other hand, continued to show lymphocytic response, but a similar response to that seen in the controls was observed in sheep 322.

5.3.2.4 *Pasteurella*-Specific Serum Antibody Responses

The *Pasteurella*-specific serum antibody responses were assayed in both indirect and double-sandwich microELISA techniques. Evaluation of whole IgG responses following primary and secondary *Pasteurella* vaccination showed that *Pasteurella*-specific antibody was detectable one week after vaccination in the uninfected sheep. This peaked between 2 and 3 weeks p.v. and was still considerably high 4 weeks p.v. at the time of secondary vaccine administration. Following booster vaccination there was a slight secondary response although this was not much higher than the primary response, probably due to the relatively short duration between the primary and booster dose administrations. However, eight weeks after booster inoculation when the experiment was terminated, the serum antibody level of these animals was still high (Figure 5.2). In contrast, in the serum of the infected sheep 445 and 479, antibody response to both primary and secondary vaccine administration was severely suppressed (Figure 5.2). On the other hand, in sheep 322 which selfcured, whilst the primary serum anti-*Pasteurella* antibody response was severely suppressed there was considerable secondary response even though the level of antibody was not as high as those of the control animals.

A panel of 3 mAbs was used in a double-sandwich ELISA to evaluate the *Pasteurella*-specific IgG1, IgG2 and IgM isotype responses after primary and secondary inoculation with *Pasteurella* vaccine. The predominant isotype produced in response to both challenges was IgG1. In the control sheep, IgG1 response

PLATE 5.1 Systemic leucocytic responses to *Pasteurella* vaccination in *T. evansi* infected and uninfected sheep.

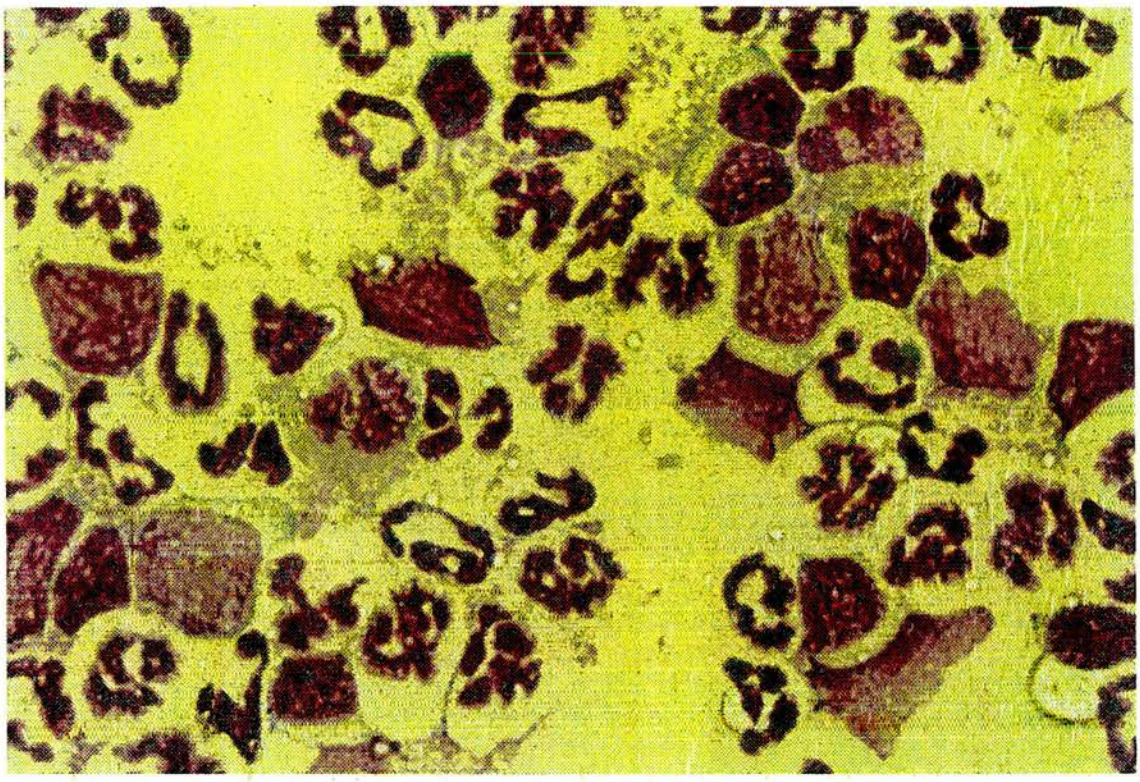
a) Presence of strong neutrophilic response after primary vaccination in normal sheep.

b) Absence of neutrophilic response after primary vaccination in infected sheep.

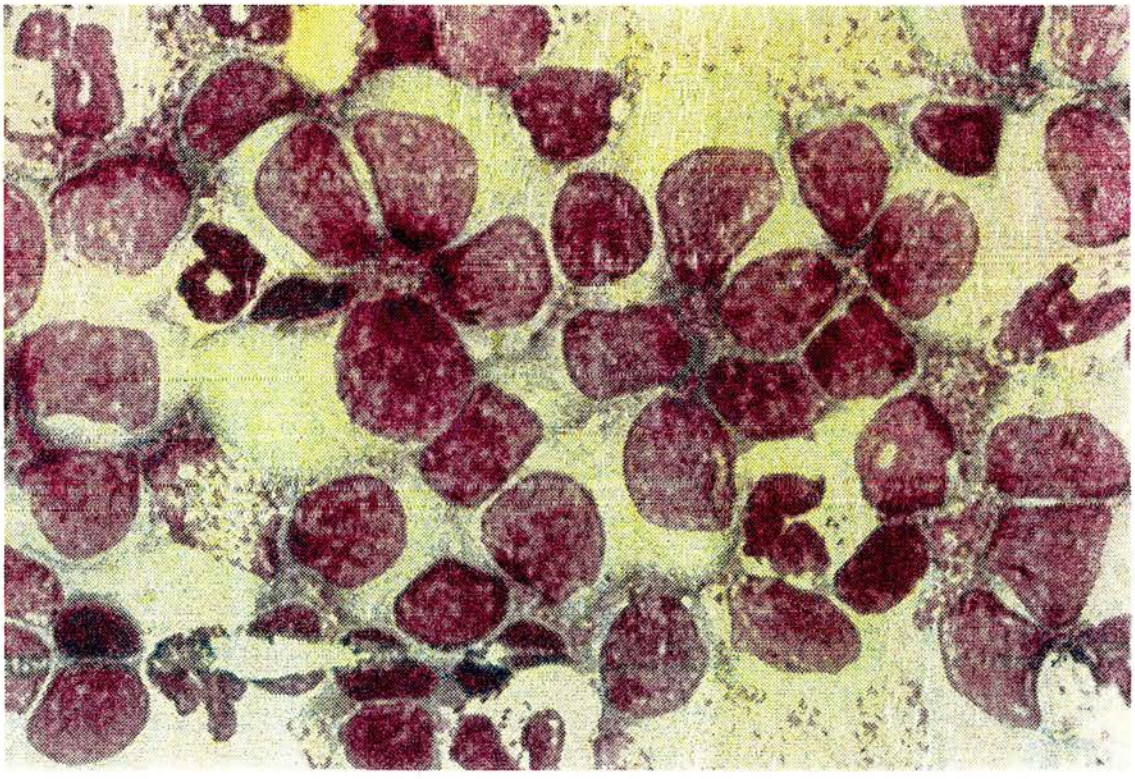
c) A mixture of lymphoblasts, small lymphocytes and neutrophils 7 days after primary vaccination in normal sheep.

d) Persistent lymphocytic blast response 7 days after primary vaccination in infected sheep.

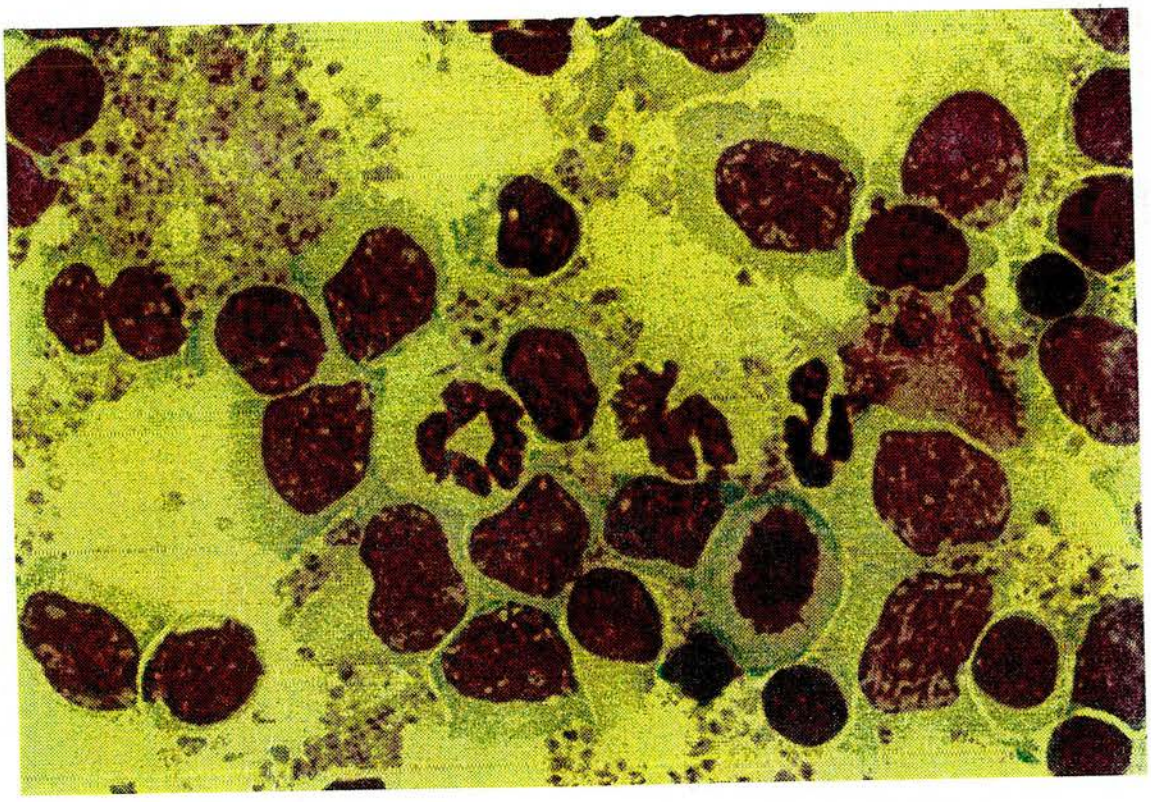
A



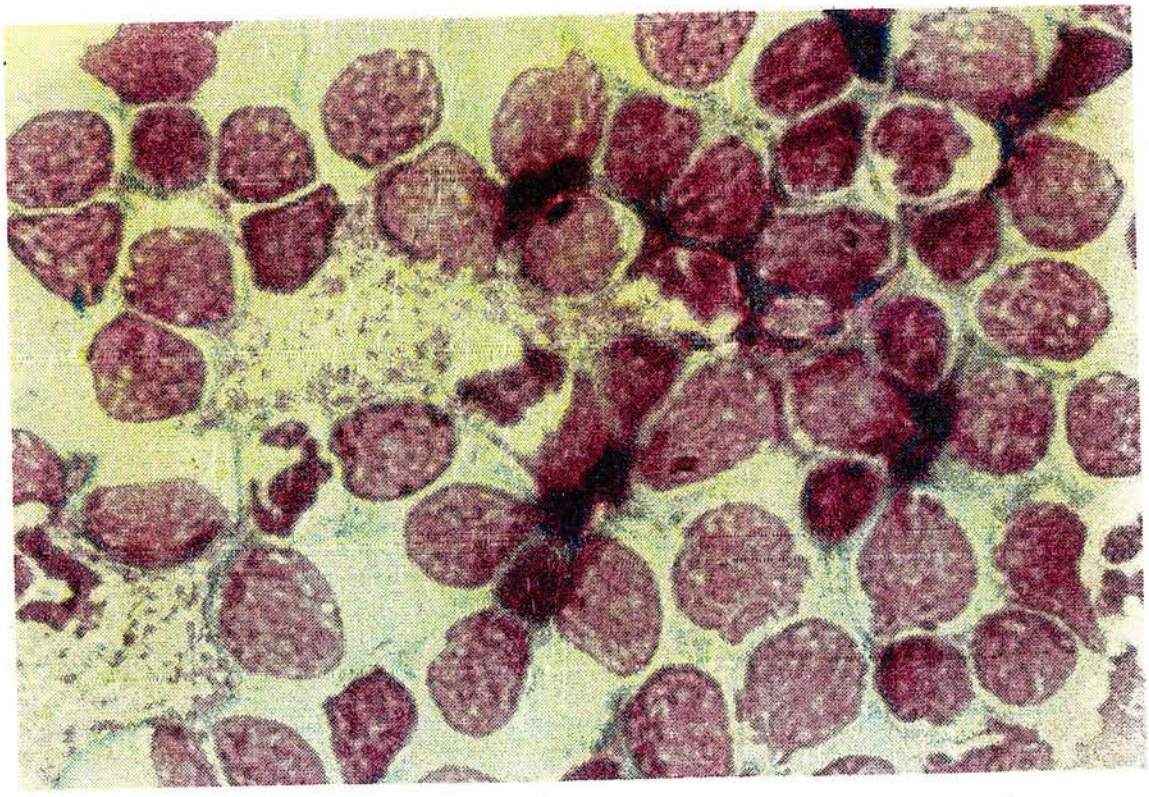
B



C



D



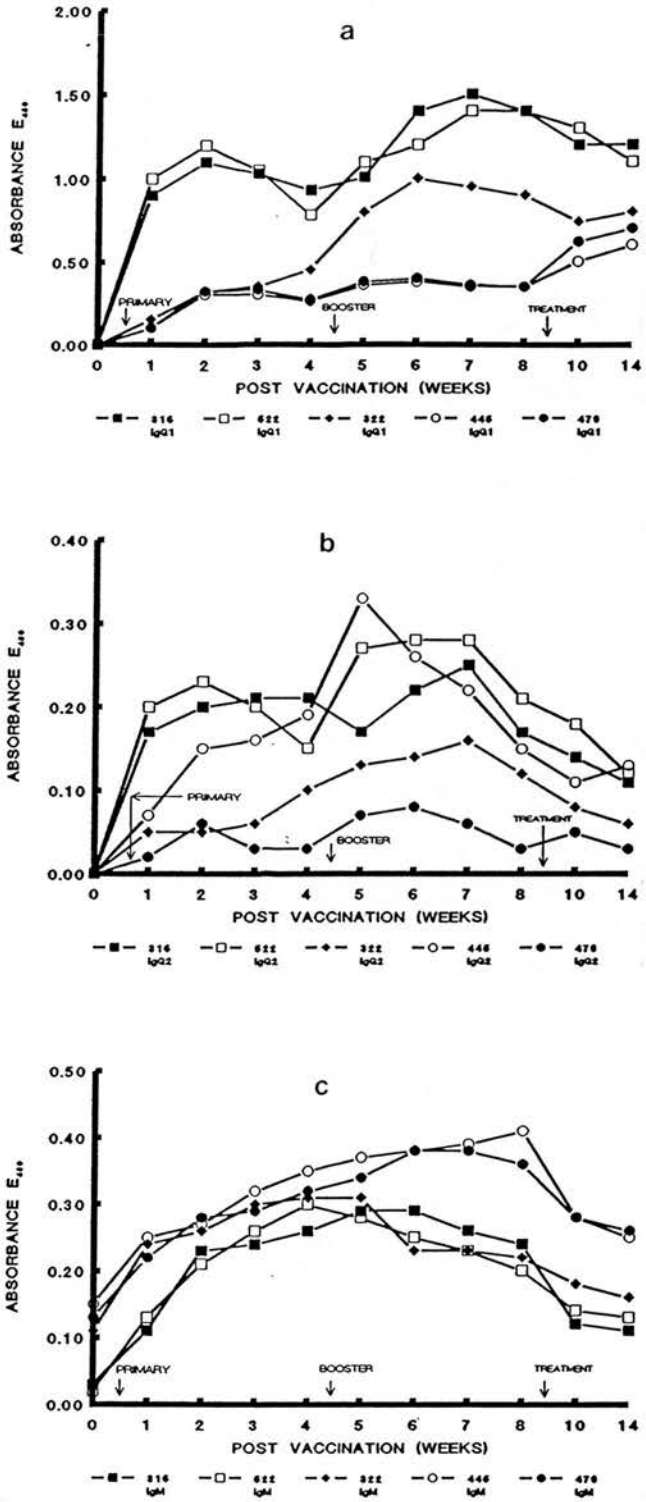


FIGURE 5.3 Isotype specific serum antibody responses to *Pasteurella* vaccination in *T. evansi* infected and uninfected sheep.
(a) IgG1; (b) IgG2; (c) IgM.

Sheep 316 and 522	=	Uninfected/vaccinated controls
Sheep 445 and 479	=	Infected/vaccinated/drug cured
Sheep 322	=	Infected/vaccinated/selfcured

peaked at two weeks p.v. and 3 weeks p.b. with the secondary response being higher than the primary (Figure 5.3a). A similar response was also seen in their IgG2 responses although only very low serum level of this antibody isotype was produced (Figure 5.3b). IgM antibodies did not show a biphasic response but rose considerably during the four weeks after primary vaccination and steadily dropped, even after the secondary booster (Figure 5.3c). In the infected sheep 445 and 479, the primary IgG1 response as well as the secondary responses were severely suppressed (Figure 5.3a). In contrast, while sheep 322 showed a depressed primary response, on booster vaccination, an enhanced secondary IgG1 response was observed, even though the level did not reach those of sheep 316 and 522. The IgG2 response differed in three infected sheep. Primary IgG2 response was suppressed in both sheep 322 and 479 whereas sheep 445 showed a continuous rise in the level until one week after boosting when it peaked at a level even higher than in the uninfected animals and rapidly declined thereafter (Figure 5.3b). The secondary response to booster dose administration remained suppressed in sheep 479 while 322 showed a considerable secondary response. The IgM response of sheep 322 was similar to those of the uninfected group. However, both 445 and 479 showed a steady rise in serum IgM levels after both primary and secondary vaccination until after trypanocidal drug treatment when the level rapidly declined (Figure 5.3c).

5.3.3 Lymphocyte Subsets in Response to Infection and/or Vaccination

5.3.3.1 Alterations in B Lymphocytes

In the uninfected sheep, there was only slight increases in the proportion and number of circulating B cells following primary and secondary *Pasteurella* vaccine administration (Table 5.2f; Figure 5.5). This is in contrast with the situation in the infected animals. In agreement with findings in Chapter 4, infection resulted in increases in cells positive for VPM 30 mAb (B cells). Following primary and secondary vaccinations, there were more than 2-fold increases in the proportion of circulating B cells in sheep 445 and 479 and this high level was maintained even 4 weeks after trypanocidal drug therapy (Table 5.2f; Figure 5.5). Sheep 322, which eventually selfcured had higher levels of circulating B cells than 445 and 479 prior to infection and vaccination. Following infection and vaccination, the increase in circulating B cells was relatively small in comparison to sheep 445 and 479 (Table 5.2f).

5.3.3.2 $CD5^+$ B Cell Expression

Two-colour indirect immunofluorescence staining using biotinylated VPM 30 (anti-B cells) and SBU-T1 (anti-CD5) mAbs showed that less than 13 percent of

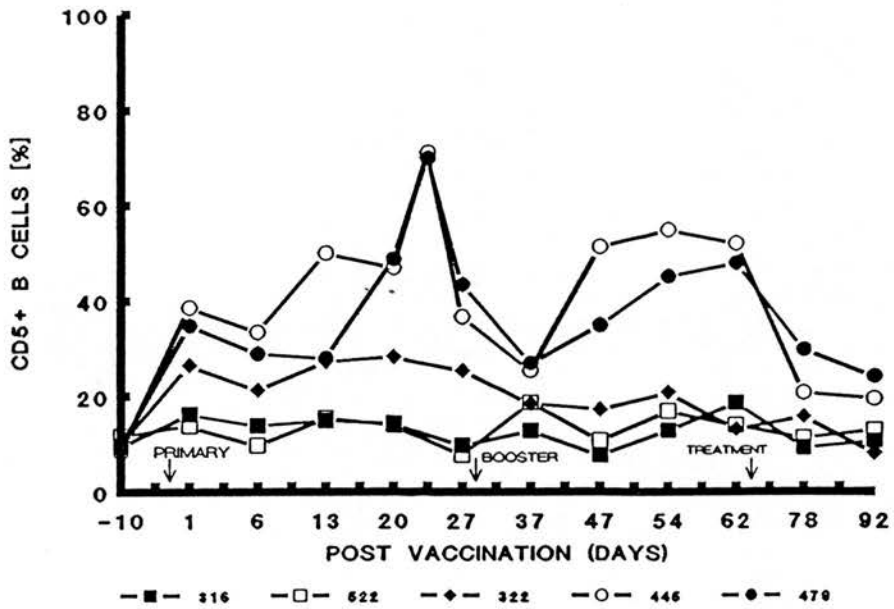


FIGURE 5.4 $CD5^+$ B cells in sheep after infection and/or *Pasteurella* vaccination.

316 and 522	=	Uninfected/vaccinated/controls
445 and 479	=	Infected/vaccinated/drug cured
322	=	Infected/vaccinated/selfcured
Primary	=	Primary vaccination
Booster	=	Booster vaccination

peripheral blood B cells bore the CD5 antigen prior to infection with *T. evansi* and/or exposure to primary and secondary *Pasteurella* vaccination. Following vaccine administration, there was a slight increase in the proportion of $CD5^+$ B cells in the uninfected sheep from a pre-vaccination level of 8.9 and 10.9 percent to 16.9 and 17.8 percent three days p.v. in sheep 316 and 522 respectively. By the second week after vaccination, the levels of B cells expressing the CD5 antigen was 14.9 and 15.5 percent respectively. After the animals were boosted the maximum proportion of $CD5^+$ B cells did not exceed 18.7 percent (Figure 5.4).

In contrast, there were marked increases in the proportion and number of $CD5^+$ B cells in the infected sheep both after *T. evansi* infection (data not shown) and following vaccine administration (Figure 5.4). During primary vaccination, whilst all infected animals showed further rises in $CD5^+$ B cells, it was higher (up to 70 percent) in sheep 445 and 479 than sheep 322 which selfcured (Figure 5.4). In these two animals, the proportion of $CD5^+$ B cells was beginning to drop by day 28 p.v., but then rose again following boosting to over 50 percent. In contrast, sheep 322 maintained a steady decline in the proportion of $CD5^+$ B cells even after the booster dose admninistration, and by day 5 p.b. the levels had become similar to those of the uninfected control sheep (Figure 5.4). Figure 5.5 shows representative FACS contour profiles of sheep 316 and 445 to illustrate the changes in $CD5^+$ B cells following *T. evansi* infection and/or primary and secondary *Pasteurella haemolytica* vaccine administration.

5.3.3.3 Alterations in MHC Class II antigen expression

Table 5.2g shows that the kinetics of MHC Class II expression in all animals was similar to those shown by B cells. Control animals showed moderate increases while increases in MHC Class II^+ cells of up to three times pre-infection levels were produced two weeks after primary vaccination in infected sheep.

5.3.3.4 Alterations in T Cell Subsets

The kinetics of the various T cell phenotypes following primary and secondary vaccine administrations differed in the uninfected and infected groups of sheep. In the control sheep, vaccination was accompanied by an initial slight decrease in the proportion of $CD5^+$ T cells. Subsequently, within two weeks of vaccine inoculation, the proportion of $CD5^+$ cells had returned to prevaccination levels or shown some increases (Table 5.2a). $CD4^+$ cells on the other hand, increased above prevaccination levels at all time points (Table 5.2b). In contrast, $CD8^+$ cells decreased after primary vaccine challenge, but following secondary administration of the vaccine, the proportions returned to prevaccination levels

FIGURE 5.5 Representative FACS contour profiles showing increase in $CD5^+$ B cells in peripheral blood of sheep infected with *T. evansi* TREU 2143. Profiles in panel A are derived from the uninfected/vaccinated sheep 316 while those in panel B are from sheep 445 which was infected and vaccinated.

In all case : Quandrant 1 = B cell population
Quadrant 2 = $CD5^+$ B cell population
Quadrant 3 = non T/non B cell population
Quadrant 4 = $CD5^+$ T cell population

Note the progressive decrease in $CD5^+$ T cells in the infected sheep (panel B,quadrant 4) in contrast to that of the uninfected sheep.

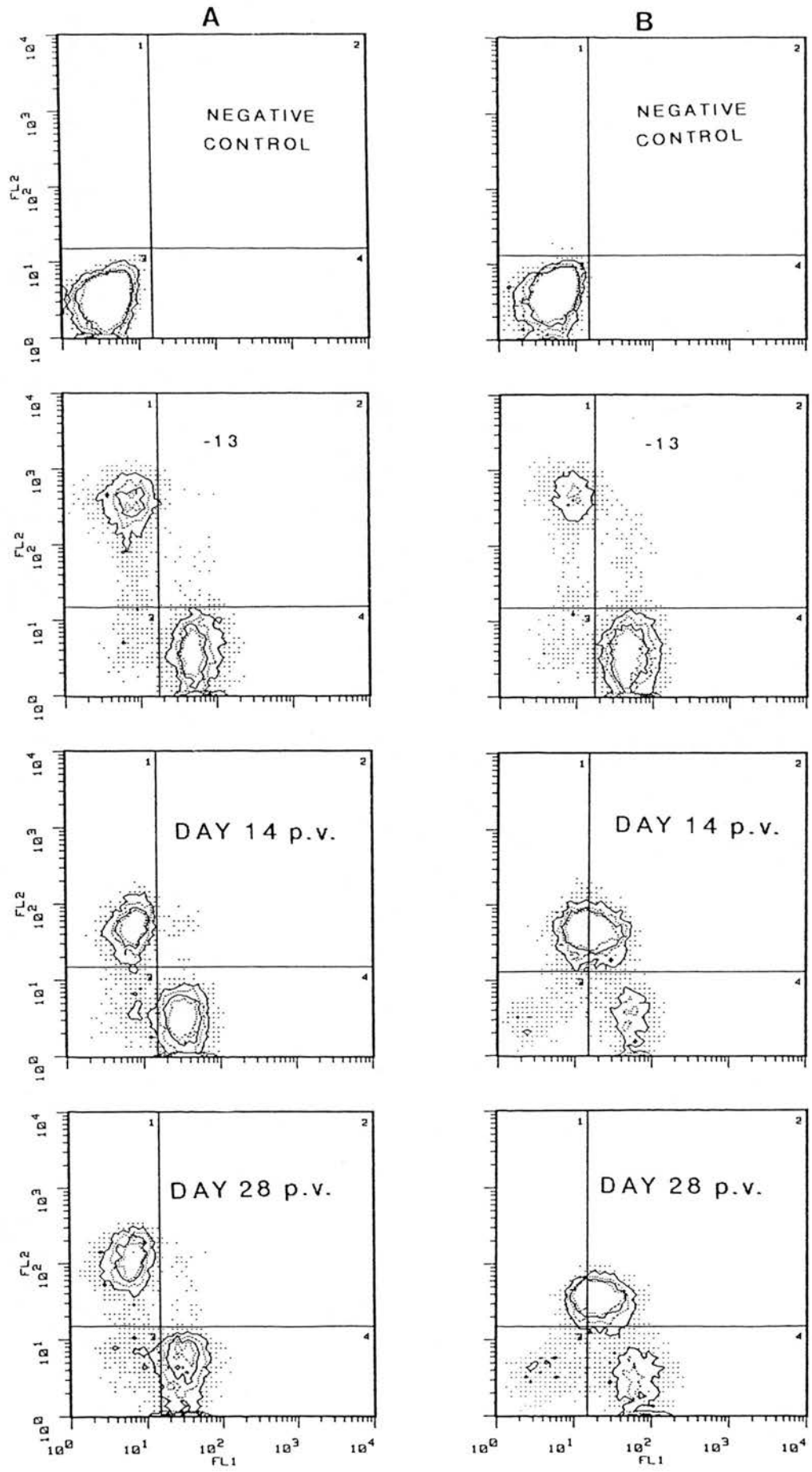

A
B
NEGATIVE CONTROL
NEGATIVE CONTROL
-13
-13
DAY 14 p.v.
DAY 14 p.v.
DAY 28 p.v.
DAY 28 p.v.
FL2
FL1
1
2
3
4

FIGURE 5.5

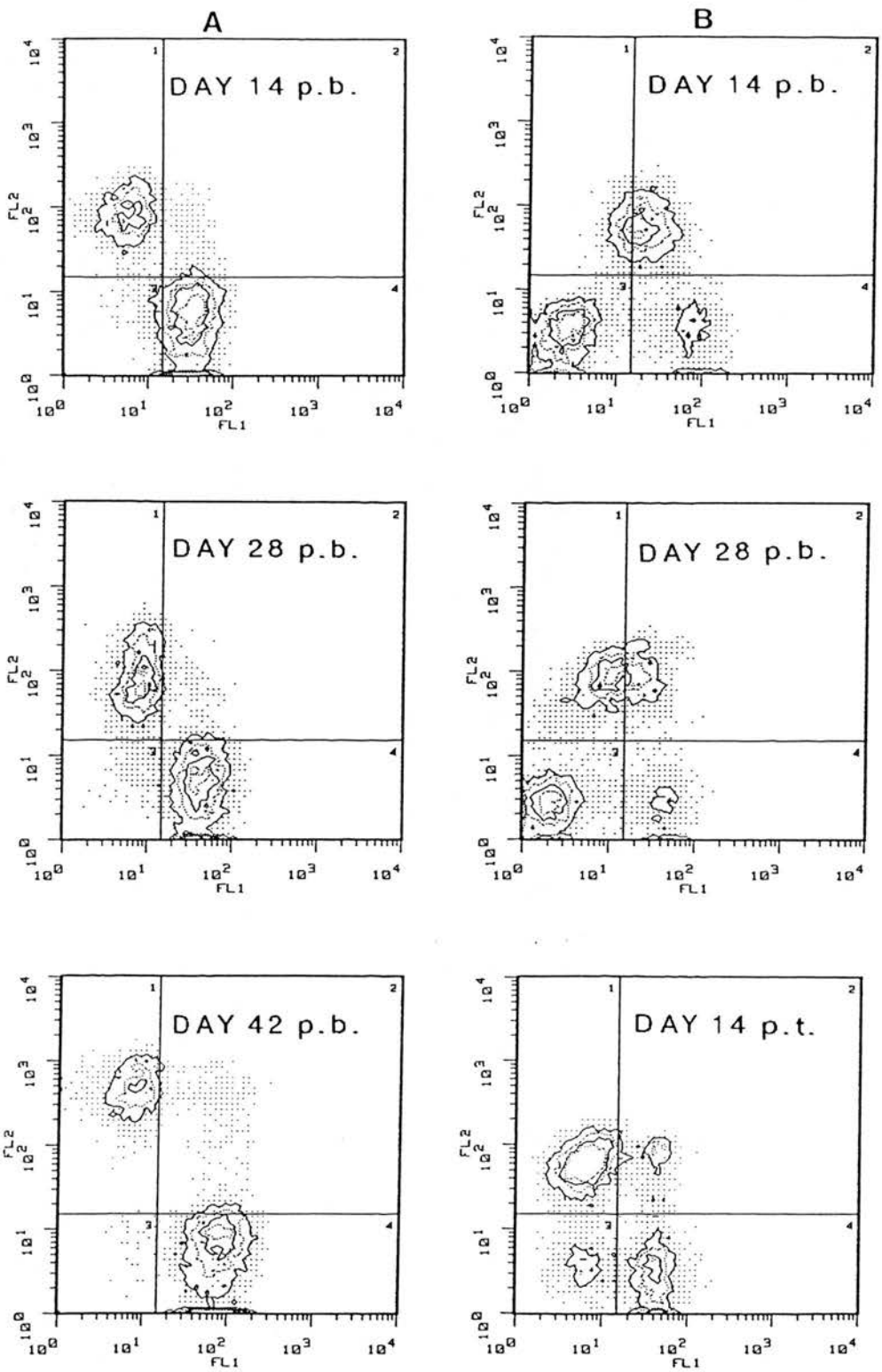

A
B
DAY 14 p.b.
DAY 14 p.b.
DAY 28 p.b.
DAY 28 p.b.
DAY 42 p.b.
DAY 14 p.t.
FL1
FL2

FIGURE 5.5

(Table 5.2c). Thus, an increase was observed in the ratio of CD4:CD8 expression as a result (Table 5.2d). There was no definite trend in the expression of γδ T cells in these animals but it generally remained within prevaccination levels (Table 5.2e).

In the infected group there were decreases in the proportions of all T cell subsets following infection and this persisted after the vaccine administration. Despite the increases in $CD5^+$ B cells, the total proportions of $CD5^+$ T cells still decreased in the infected sheep. This decrease was less marked in sheep 322. For instance, the proportion of $CD5^+$ cells in the infected sheep 445 and 479 decreased from a pre-infection mean of 67.8 percent to 34.4 and 37.5 percent in the second week following primary and secondary vaccine inoculations respectively (Table 5.2a; Figure 5.5). In contrast, only a 12 and 14 percent decrease from a pre-infection value of 46 percent to 34 and 32 percent respectively were seen during the same periods in sheep 322 (Table 5.2a). Similarly, there was a greater decrease in $CD4^+$ cells in sheep 445 and 479 than in sheep 322 (Table 5.2b) but the decreases in $CD8^+$ and γδ T cells were similar in all three sheep (Table 5.2c, d).

5.4 Discussion

It is apparent that primary and secondary cellular and humoral immune responses to inoculation with *Pasteurella haemolytica* serotype A1 vaccine were suppressed in sheep infected with *T. evansi*. Both cellular and humoral immune responses are required for effective protective immunity against pneumonic pasteurellosis (Confer *et al.*, 1988). Cellular immunity involves the mobilisation of neutrophils and cells of the monocyte/macrophage lineage which ingest and kill opsonised *Pasteurella* organisms. It has been shown that factors which impair this mobilisation usually result in hypersusceptibility to pasteurellosis (Noel *et al.*, 1988). It was clearly shown in the present study, that *T. evansi* inhibited systemic mobilisation of neutrophils and local erythematous skin response in infected sheep and that the sheep subsequently failed to produce significant levels of anti-*Pasteurella* antibody. It is not clear how *T. evansi* infection inhibited the local erythematous induration at the site of vaccination or the systemic mobilisation of neutrophils observed in the control sheep. However, since the secondary responses were partially restored in sheep 322 which had selfcured by the time of the secondary vaccine inoculation, active infection is a necessary condition for the suppression. Suppression of cell-mediated DTH response to PPD associated with active trypanosome infection has been reported in rabbits and rats infected with *T. congolense* and *T. brucei* respectively (Mansfield and Wallace, 1974; Mackenzie, Sibley and White, 1979). Moreover, both the formation of a local erythematous induration at the site of vaccine administration and systemic mobilisation of

Table 5.2 **Changes in the proportions of peripheral blood leucocyte subsets in sheep infected with *T. evansi* and/or inoculated with *P. haemolytica* vaccine.**

Sheep 316 and 522	= Uninfected/vaccinated controls
Sheep 322	= Infected/vaccinated but underwent self cure prior to secondary vaccination
Sheep 445 and 479	= Infected/vaccinated. Remained parasitaemic until treated with a trypanocidal drug.
p.v.	= post vaccination

(a) $CD5^+$ cells (%)

Weeks	316	522	322	445	479
-2	51.8	44.3	41.4	64.4	71.2
-1	50.5	42.9	46.1	64.7	71.8
		Primary vaccination			
1	48.7	41.6	30.9	45.9	42.8
2	55.9	44.6	34.4	38.8	30.1
3	59.6	47.2	39.4	45.4	50.6
4	56.6	47.0	46.3	44.3	59.7
		Secondary vaccination			
1	50.4	44.4	34.8	40.9	53.6
2	64.3	45.4	32.2	36.3	38.7
3	65.5	49.0	40.6	44.4	55.6
4	60.3	44.2	37.6	55.8	53.9
		Trypanocidal drug treatment			
2	52.2	41.7	41.8	46.8	52.3
4	54.9	42.6	43.2	58.3	58.6

(b) $CD4^+$ cells (%)

Weeks	316	522	322	445	479
-2	20.1	15.2	17.3	36.8	34.5
-1	20.7	15.2	16.1	36.9	34.9
		Primary vaccination			
1	25.7	17.5	11.5	18.9	13.7
2	28.1	17.7	11.5	14.9	12.1
3	23.3	19.1	13.8	15.4	17.9
4	24.3	18.8	13.9	18.4	19.2
		Secondary vaccination			
1	22.9	17.9	13.1	16.0	21.3
2	29.0	17.8	11.1	11.4	16.3
3	28.2	17.8	13.7	15.3	19.3
4	24.2	15.7	11.5	17.4	16.4
		Trypanocidal drug treatment			
2	20.8	17.7	15.7	26.9	25.1
4	20.8	15.1	14.0	29.2	23.5

(c) $CD8^+$ cells (%)

Weeks	316	522	322	445	479
-2	18.2	13.7	16.5	15.1	17.8
-1	18.0	13.5	15.2	14.9	18.4
		Primary vaccination			
1	18.6	9.3	8.1	9.7	7.7
2	16.2	8.6	8.1	6.7	7.0
3	16.4	12.6	8.6	7.7	8.9
4	14.7	11.6	9.1	9.7	10.7
		Secondary vaccination			
1	18.5	13.7	9.6	7.8	11.0
2	19.3	13.6	7.4	6.7	7.8
3	20.7	16.5	9.4	12.3	17.0
4	19.0	14.6	7.9	8.4	11.9
		Trypanocidal drug treatment			
2	17.3	13.1	13.7	8.5	13.1
4	18.8	9.7	13.8	11.5	14.3

(d) CD4:CD8 ratio

Weeks	316	522	322	445	479
-2	1.1	1.1	1.0	2.4	1.9
-1	1.2	1.1	1.1	2.5	1.9
		Primary vaccination			
1	1.4	1.9	1.4	1.9	1.8
2	1.7	2.1	1.4	2.2	1.7
3	1.4	1.5	1.6	2.0	2.0
4	1.7	1.6	1.5	1.9	1.8
		Secondary vaccination			
1	1.2	1.3	1.4	2.1	1.9
2	1.5	1.3	1.5	1.7	2.1
3	1.4	1.1	1.5	1.2	1.1
4	1.3	1.1	1.5	2.1	1.4
		Trypanocidal drug treatment			
2	1.2	1.4	1.1	3.2	1.9
4	1.1	1.6	1.0	2.5	1.6

(e) γδ T cells (%)

Weeks	316	522	322	445	479
-2	9.4	7.4	9.8	7.2	11.7
-1	8.2	6.2	9.3	8.8	11.0
		Primary vaccination			
1	8.6	8.2	3.7	4.0	5.1
2	8.0	7.5	4.1	2.5	4.7
3	9.2	6.3	5.3	2.8	3.6
4	12.8	5.7	5.7	3.2	5.0
		Secondary vaccination			
1	9.9	7.7	3.9	2.0	4.9
2	10.3	7.8	4.2	2.4	6.2
3	9.1	6.1	4.3	6.9	6.5
4	8.9	8.6	4.2	5.6	6.9
		Trypanocidal drug treatment			
2	10.7	7.0	7.3	3.6	9.7
4	10.4	8.2	5.8	6.5	8.8

(f) VPM 30^+ cells (%)

Weeks	316	522	322	445	479
-2	40.7	39.5	45.9	25.9	26.0
-1	32.7	40.7	47.5	26.9	21.4
		Primary vaccination			
1	38.5	42.6	63.6	62.8	523.5
2	40.0	47.1	66.0	68.2	60.5
3	44.8	46.6	57.3	62.2	54.3
4	42.7	45.2	59.6	60.5	48.4
		Secondary vaccination			
1	43.1	46.4	56.6	53.1	43.8
2	40.1	44.2	56.9	57.7	58.4
3	44.2	48.2	61.1	50.2	58.8
4	45.3	42.7	57.5	59.4	51.9
		Trypanocidal drug treatment			
2	33.2	49.7	48.2	56.0	47.5
4	35.8	49.5	53.7	47.4	48.5

(g) MHC II^+ cells (%)

Weeks	316	522	322	445	479
-2	41.7	50.6	45.8	24.4	41.3
-1	37.8	51.2	47.4	26.8	39.1
		Primary vaccination			
1	45.3	57.1	66.3	68.6	67.0
2	47.0	56.0	72.4	76.0	70.3
3	55.0	58.8	68.2	70.1	70.0
4	44.7	50.8	69.3	63.3	60.3
		Secondary vaccination			
1	44.8	53.2	61.3	50.1	53.5
2	41.4	54.2	67.4	40.6	67.2
3	42.4	51.9	63.9	44.6	58.7
4	52.1	60.3	66.3	61.0	69.1
		Trypanocidal drug treatment			
2	52.8	55.1	62.7	60.5	57.3
8	49.9	51.9	60.4	47.4	48.5

neutrophils, require the production of the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3. Both of these act on the bone marrow for the mobilisation and maturation of inflammatory cells (Stadnyk and Gauldie, 1991). These cytokines are products of $CD4^+$ and $CD8^+$ T cells (Mosmann and Moore, 1991), both of which were markedly reduced in proportion by the *T. evansi* infection. Thus, it is possible that the suppressed local skin response and systemic neutrophilic responses to the vaccine in the infected/immunised sheep may in part have resulted from a reduced level of cytokine release by these T cell phenotypes.

The assay for *Pasteurella*-specific antibody responses showed that in the uninfected control sheep IgG1 antibody was the predominant antibody produced. This is in agreement with previous reports in which IgG1 antibody was shown to be the most important antibody class mediating protection in pasteurellosis (Donachie *et al.*, 1986; O'Brien and Duffus, 1987; Mosier *et al.*, 1989; Nelson and Frank, 1989). In contrast, in sheep with a 28-day old *T. evansi* infection, both primary and secondary humoral responses to *Pasteurella* vaccine were suppressed and this suppression particularly affected IgG1 levels. There are virtually no studies of *T. evansi* induced suppression of immune responses to vaccine antigens in livestock except for that of Shien (1979), in which the humoral antibody response to *Brucella abortus* S-19 vaccinations in goats infected with *T. evansi* was found to be significantly suppressed. That report, in addition to the present study, shows that *T. evansi* is capable of inducing generalised immunosuppression similar to that induced by the tsetse transmitted trypanosomoses.

The IgG2 response in sheep 445 was unusual since it was higher than that observed in the other infected sheep as well as the uninfected sheep. In mice, IgG2 production by B cells is mediated by the T_H1 subset of the $CD4^+$ T cells through its synthesis of IFN–γ (Snapper and Paul, 1987; Coffman *et al.*, 1988). Moreover, IFN-γ on the other hand suppresses the production of IgG1 by B cells through its potent inhibition of T_H2 proliferation and growth (Coffman and Carty, 1986; Mosmann and Moore, 1991). Sheep 445 had relatively the highest parasitaemia of all the infected sheep. Since increased parasitaemia is associated with increased levels of IFN-γ and activation of macrophages (Bancroft *et al.*, 1983; Grosskinsky *et al.*, 1983; Bakhiet *et al.*, 1990), it is possible that the higher IgG2 response may have resulted from higher IFN-γ production by $CD4^+$ and $CD8^+$ T cell subsets.

Although IgM antibody was detected in all 5 sheep following primary vaccine administration its importance as an anti-*Pasteurella* antibody is not clear. It has been shown that low levels of IgM are detected following exposure to *Pasteurella* antigen but it does not play any major role as a specific protective anti-*Pasteurella*

antibody (O'Brien and Duffus, 1987; Mosier *et al.*, 1989) and therefore has no major role in resistance against the disease. It is noteworthy that there was no difference in the IgM response in all 5 sheep following primary exposure to the vaccine and that while the level steadily declined, even after boosting, in 316 and 522 as well as in 322 which selfcured, it continued to rise in sheep 445 and 479 until after trypanocidal drug treatment. Moreover, the results revealed that much higher IgM antibody levels were already detectable in the serum collected from these animals following trypanosome infection and prior to administration of the *Pasteurella* vaccine. This means that not all the IgM produced in infected/vaccinated sheep could have been elicited specifically by the vaccine antigen. This is not entirely surprising since trypanosome infections are known to induce an IgM hyper-gammaglobulinaemia resulting in production of heterophile antibodies which react with a variety of other antigens (Henderson-Begg, 1946; Houba, Brown and Allison, 1969; reviewed in Parratt and Herbert, 1979).

In the control sheep, alterations in T cell subsets were relatively minor and involved an initial transient drop in $CD5^+$ cells. This drop was a reflection of the increase in the percentage of circulating neutrophils during that period which also resulted in a transient decrease in the relative proportion of circulating lymphocytes. The slight rise observed subsequently in the proportion of $CD5^+$ cells did not result from increased expression of $CD5^+$ B cells since the proportion of this B cell population after vaccination did not differ from the prevaccination values. In addition, a marginal increase in CD4:CD8 ratio in the control sheep following vaccination occurred as a result of a slight increase in $CD4^+$ cells. This differs from the rises seen in infected, selfcured sheep in which the increase occurred as a result of a greater reduction in the proportion of the $CD8^+$ cells. However, regardless of the manner of this increase, results of the trypanosome- and *Pasteurella*-specific antibody assays conducted in these experiments seem to indicate that a higher proportion of $CD4^+$ cells, no matter how marginal, is necessary for a more effective specific antibody response against the trypanosomal and *Pasteurella* antigens.

In the infected animals, infection resulted in decreases in the proportions of all the T cell subsets similar to those described in Chapter 4. Decreases in the percentage of $CD5^+$ T cells did not result in decreases in the absolute number of cells expressing this antigen. This was accounted for by the expansion in the number of $CD5^+$ B cells. After vaccination however, the $CD5^+$ T cells decreased dramatically both in proportions and absolute numbers despite the increase in $CD5^+$ B cell proportion and numbers. It is not clear why vaccination exacerbated the selective depletion of the $CD5^+$ T cell subset in the two infected sheep (445 and 479). One possible reason could be the massive expansion in the B cell population.

However, the two-colour immunofluorescence staining of B cells and T cells for CD5 antigen clearly showed that infection and vaccination resulted in progressive reduction in the proportion of $CD5^+$ T cells, in line with the decrease in the percentage of other T cell phenotypes. Hypothetically, it is possible that vaccination of the infected animals resulted in a hyperactivation of mature T cells which drove them to lose their surface antigens. Several studies have produced evidence that CD3, CD4 and CD8 antigens may be lost from mature human and murine T cells upon activation *in vitro* (Bensussan *et al.*, 1985; Cantrell, Davies and Crumpton, 1985; Hoxie *et al.*, 1986; Isakov *et al.*, 1987; Takada and Engleman, 1987; Weyand, Goronzy and Fathman, 1987). Moreover, *in vivo* as well as *in vitro*, activation of mature peripheral T cells in sheep results in the loss of the CD5 antigen (Hopkins and Dutia, 1989). However, the loss of surface antigen expression *per se* does not account for the terminal decrease in the number of circulating T lymphocytes, and the accompanying immunological malfunction. It has been suggested that single positive mature T cells shift to a transient double positive state upon activation (Blue *et al.*, 1985). Co-expression of CD4 and CD8 (double positive, DP) is characteristic of immature thymocytes, which mature into single $CD4^+$ or $CD8^+$ T cells or are eliminated prior to leaving the thymus (Blackman, Kappler and Marrack, 1990). Outside the thymic environment cells exhibiting immature characteristics are eliminated by a normal physiological process known as programmed cell death or apoptosis (Ameisen and Capron, 1991; Young, 1992). If trypanosome infection induces T cells to co-express the CD4 and CD8 antigens upon activation, or to mobilise immature $CD4^+CD8^+$ cells from the thymus, then it might prime them for destruction by apoptosis. This could explain the reduction in the proportion of circulating T cells. However this hypothesis is speculative and was not investigated further. The decrease in peripheral T cell proportions may well have arisen from their depletion in the T cell dependent areas in the lymphoid organs. In longstanding trypanosome infections in cattle the paracortical areas of the lymph nodes become depleted of cells (Morrison and Murray, 1979). Similarly, the T cell-dependent areas of lymph nodes draining local skin reactions at site of metacyclic *T. congolense* infection in sheep are depleted of small lymphocytes (Mwangi *et al.*, 1991). Moreover, the large reductions in the proportions of T cell phenotyes is also partly a reflection of the marked B cell lymphocytosis observed in the infected animals.

Analysis of B cells in the infected sheep revealed large increases in the proportions and numbers of circulating B cells in sheep 445 and 479. This increase was partly due to the considerable increase in the $CD5^+$ B cell population which appeared early in infection and persisted after vaccination. The underlying

mechanisms behind the *T. evansi*-induced increase in $CD5^+$ B cells is not clear. However, similar increases in $CD5^+$ B cells have been reported in cattle infected with *T. congolense* where between 49-90 of B cells express the CD5 antigen by day 19 p.i. (Williams *et al.*, 1991). Moreover, infection of mice with *T. cruzi* also led to the expansion of $CD5^+$ B cells. These cells were implicated in the generation of immunoglobulins that were non-restrictive, did not recognise specific parasite antigens and reacted with self components instead (Minoprio *et al.*, 1988; 1989a, b; Spinella *et al.*, 1990; Minoprio, 1991). Increases in $CD5^+$ B cells have also been reported in other chronic diseases presenting with disorders of the immune system. For instance, this B cell phenotype is increased in cattle infected with bovine leukaemia virus (Depelchin *et al.*, 1989), in human patients suffering from rheumatoid arthritis (Burastero *et al.*, 1988) and systemic lupus erythmatosus (Casali *et al.*, 1987; 1989) and in autoimmune mice (Hayakawa *et al.*, 1983). It would appear that the production of high levels of non-specific IgM observed in trypanosome infection is a result of the expanded $CD5^+$ B cell population since this cell phenotype is known to produce poly-reactive, low affinity IgM (Casali and Notkins, 1989). In the course of trypanosome infection, the persistence of $CD5^+$ B cells and their action is compounded by the marked reduction in the proportion of $CD4^+$ and $CD8^+$ cells. Thus antigen-specific help by $CD4^+$ cells to conventional B cells is compromosed as well as the regulation of immune responses by $CD8^+$ cells. However, since these reductions in $CD4^+$ and $CD8^+$ T cells were minimal in terms of their absolute numbers, it is probable that other mechanisms also operate to undermine the immune competence of infected animals. In any case, that these alterations in the proportions of various cell phenotypes account, at least in part, for the failure of infected animals to mount an efficient immunological response to the vaccine antigen or even to the parasite antigens is supported by the response of the selfcured sheep. For instance, there was a minor rise in both the conventional B cells and in $CD5^+$ B cells in sheep 322 which selfcured. This animal, as well as others which selfcured, showed alterations in the proportions of their $CD5^+$, $CD4^+$ and $CD8^+$ cells such that an increase in the CD4:CD8 ratio was observed. The net result of this was that not only did they rid themselves of the trypanosome infection, but sheep 322 was also able to respond to secondary *Pasteurella* vaccine administration at a level which approximated those of the uninfected control sheep. These results also showed that the proportionate reduction in T cell subsets and increased mobilisation of conventional B cells and $CD5^+$ B cell population was not simply an index of lymphocyte activation by antigen since they were not precipitated by primary and booster *Pasteurella* vaccinations in the control sheep.

CHAPTER SIX

Changes in cell dynamics and phenotypes in efferent lymph draining the site of *Pasteurella* vaccination

6.1 Introduction

Sheep infected with *T. evansi* show considerable alterations in the proportions of circulating lymphocytes in the peripheral blood, in particular, pronounced increases in B cells, decreases in T cell phenotypes and increased expression of the CD5 antigen on B cells (Chapters 4 and 5). It is likely that these changes play a key role in compromising the defence mechanisms of infected animals against both the invading parasites and other heterologous antigens. However, the blood is only a part of the mammalian lymphoid system and although it serves as a traffic route for circulating lymphocytes, immunological events occurring in it may not reflect the cellular changes taking place immediately upon the introduction of an antigen via the intradermal or subcutaneous portal of entry. It is the nature of early interaction between the pathogen and hosts effector cells which determine the outcome of the infection. Ineffectual responses result in disease whilst strong responses render the pathogen innocuous. Such early local changes in individual cell populations or expression of immunologically important cell surface molecules are best studied by chronically cannulating lymphatic ducts draining the site of infection and collecting lymph from the fully conscious animal. It is then possible to assess whether cellular changes such as occur in the blood also occur in the lymphatic system of infected animals and to determine the immunological implications.

A number of studies conducted in sheep have shown that the early immunological events occurring in lymph nodes draining sites of antigen deposition and inflammatory lesions are reflected by the cell content of the efferent lymph draining that node (Trnka and Cahill, 1980; Hopkins, Dutia and McConnell, 1986; Bujdoso *et al.*, 1989). For instance, the cellular composition of efferent lymph draining unstimulated lymph nodes is entirely of small lymphocytes, consisting of approximately 30 percent B cells and 70 percent T cells (Hopkins *et al.*, 1985). However, following an antigenic stimulus, the cell content and composition changes, resulting in an increased output of small lymphocytes and lymphoblasts and in some circumstances may include a mixture of erythrocytes and granulocytes (Hall and Morris, 1962; 1965a, b; McColgan, Buxton and Miller, 1987; Kerlin and Watson, 1987; Huang *et al.*, 1991; Mwangi, 1991). Moreover, the changes in cellular composition is also accompanied by kinetic changes in the expression of cell surface molecules by the efferent lymphocytes. The nature and trend of these kinetic changes depend on the type of antigenic stimulus. For instance, in sheep, following stimulation of the node with ovalbumin [OVA] or purified protein derivative [PPD] of tuberculin, the increased cell output in the draining efferent lymph is accompanied by an increase in the percentages of $CD4^+$ and $CD8^+$ cells and in T

Table 6.1 **Summary of experimental design and duration of experiments after surgical cannulation of prefemoral efferent duct in sheep**

Sheep	Infection status (2×10^6)	Surgery performed (days p.i.)	Number of Surgeries	Day post surgery when sample collection for analysis began	*Pasteurella* vaccination		Duration of analytical Sample collection (days)			
					Primary	Secondary	Pre-vaccine	Post vaccine	Post booster	Total
	T. evansi									
662	TREU 2143	10	2	5	7 d.p.c.	4 d.a.s.c.	2	14	12	28
669	"	10	2	5	"	"	2	14	7	23
1218	"	10	1*	NIL	NIL	NIL	NIL	NIL	NIL	NIL
1223	"	23	1	5	5 d.b.c.	15 p.v.++	NIL	5	NIL	5
200	NIL	+	2	5	7 d.p.c.	4. d.a.s.c.	2	12	5	19
569	"	+	1	5	"	12 p.v.	2	12	12	26
664	"	+	2**	5	"	NIL	2	12	NIL	14
1224	"	+	1	5	5 d.b.c.	15 p.v.	NIL	5	8	13

\+ Cannulated on the same day as the infected sheep
* Not successful as duct could not be located
** Cannula pulled out 4 hours after the second surgery
d.p.c. days post cannulation
d.b.c. days before cannulation
d.a.s.c. days after second cannulation
p.v. post vaccination
++ Lymph flow ceased after booster

cells expressing the MHC Class II antigen (Hopkins *et al.*, 1986; Bujdoso *et al.*, 1989). Similarly, in sheep persistently infected with border disease virus (BDV) or those infected with the orf virus or *Chlamydia psittaci*, there is increase in cells expressing the T cell-associated molecules CD5, CD4, CD8 and T19, and also increases in cells expressing CD1 and MHC Class II molecules (Huang *et al.*, 1991; Yirrell *et al.*, 1991; Entrican *et al.*, 1992).

In contrast, in efferent lymph of prefemoral lymph node draining the site of infection of sheep with culture-derived metacyclic *T. congolense*, an initial decline in the various T cell subsets is followed by a transient increase before steadily declining again; while B cells as well as MHC Class II positive cells show marked increases (Mwangi, 1991). These findings are similar to observations made in the peripheral blood leucocyte subsets in cattle and sheep infected with *T. congolense* or sheep infected with *T. evansi* (Ellis *et al.*, 1987; Mwangi, 1991; Williams *et al.*, 1991; Chapters 4 and 5). Thus, it appears that alterations in lymphocyte subsets engendered by trypanosome infections is not restricted, but affects other compartments of the lymphoid organ system. Since cells leaving the efferent lymph of an antigen primed lymph node are responsible for the systemic dissemination and amplification of the local immune response it is important that such primed cells leave the node expressing their immunologically important effector molecules in adequate numbers and proportions as are necessary for effective immunity.

To investigate further the effects of *T. evansi* on the immunological events taking place in infected sheep experiments were conducted to study the cellular demographics of efferent lymph draining the site of *Pasteurella haemolytica* vaccine administration in *T. evansi* infected sheep.

6.2 Materials and Methods

6.2.1 Animals, Infection and Vaccination

Six, 6-month old Finnish Landrace sheep and two 8-month old Suffolk sheep were used in this study. All the animals were housed and fed as described in Section 3.1. Prior to the experiments all the animals tested negative for significant anti-*Pasteurella* antibody in an ELISA system. Four of the sheep were infected with *T. evansi* as described in Section 3.2.3, 10 or 23 days prior to cannulation (Table 6.1). The sheep were each primed by s.c. injection of a 2 mg dose of *Pasteurella* vaccine in the draining area of the prefemoral lymph node on the days specified in Table 6.1. For the infected sheep this corresponded to 17 days post infection, at which time they had all become parasitaemic by HCT.

6.2.2 Surgery and Lymph Collection

The cannulation of the efferent lymphatic duct of the prefemoral lymph node was according to the technique of Hall and Morris (1965, 1967), and was briefly described in Section 3.6. In some animals lymph flow ceased before the secondary vaccination. It was therefore necessary in such animals to cannulate the contralateral duct for secondary vaccine challenge and data collection. In such cases, animals were allowed six weeks of recovery before the second operation. All cannulations were allowed to stabilise for 5 days following surgery before lymph collection for analytical purposes was begun. Lymph was collected quantitatively as described in Section 3.6.2. Each collected lymph was processed to obtain volume, cell count, total cell output and blast cell numbers. Trypanosome numbers in the lymph were determined using a haemocytometer when possible.

6.2.3 Phenotypic Analysis of Efferent Lymphocytes

Indirect immunofluorescence staining and flow cytometry described in Sections 3.5.3 and 3.5.2 respectively were used to analyse phenotypic changes in efferent lymphocytes following i.v. infection of sheep with *T. evansi* and/or *in vivo* stimulation of the lymph node with *Pasteurella* vaccine. S-T8 and biotinylated SBU-T4, both visualised as described under 2-colour analysis of cell phenotypes in Section 3.5.3, were used to monitor the coexpression of CD4 and CD8 antigen on efferent lymphocytes derived from the primed lymph node. The expression of CD5 antigen on efferent lymph B cells was similarly monitored using the anti-sheep CD5 mAb, SBU-T1 and biotinylated VPM 30 (anti-sheep B cell) mAb. The mAb SBU-II (anti-sheep MHC Class II) was used with VPM 30-biotin to assay Class II expression on B cells. In addition γδ, CD1, CD45 and MHC Class I expression by the cells was monitored using SBU-T19, SBU-T6, SBU-LCA and SBU-I mAbs respectively.

6.3 Results

Eight animals, four infected with *T. evansi* TREU 2143 and four uninfected controls, were used to study the effects of infection on the cellular demographics in efferent lymph of the lymph node draining the site of *Pasteurella* vaccine administration. Cannulations of the prefemoral efferent duct was successful in seven sheep but flow of lymph for a sufficiently long period to permit collection of samples for pre- and post-primary and secondary vaccination analysis was successful in only two infected and two uninfected sheep (Table 6.1). The results reported here are therefore based on observations made on these four animals. Because the two infected animals showed similar trends in their responses and also because of the similarity in the responses of the uninfected sheep, the results are

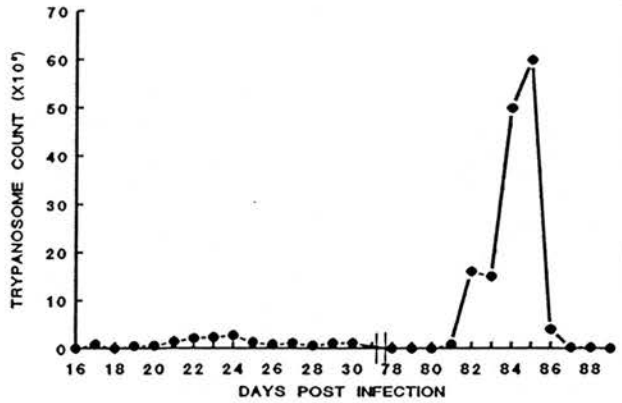


FIGURE 6.1 Parasite kinetics in the efferent lymph of sheep infected intravenously with 2 x 10^6 *T. evansi* ml^{-1}. Figures represent mean counts per ml of lymph from two sheep. Two peaks were observed. The first peak was relatively minor and occurred between days 22 to 24 p.i. when approximately 3 x 16^6 parasites ml^{-1} were detected. The second peak occurred between days 82 to 86 p.i. when up to 6 x 10^7 parasites ml^{-1} of lymph was detected, a 22-fold increase in parasitosis. This larger increase may be a reflection of depressed immune competence of the sheep.

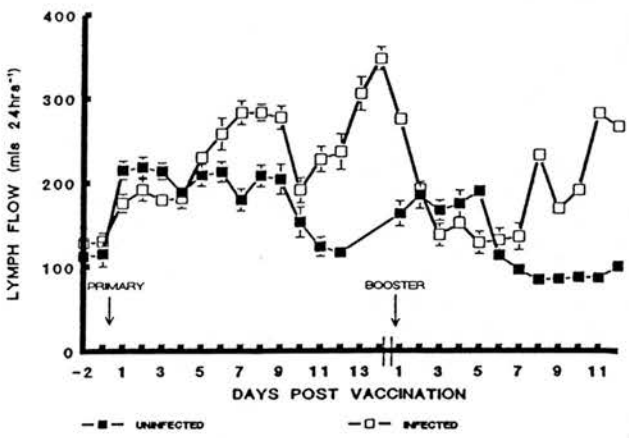


FIGURE 6.2 Daily lymph output in sheep after infection with *T. evansi* and/or vaccination with *P. haemolytica*. Values represent Means ± S.D. of two infected/vaccinated and two uninfected/vaccinated sheep. In the infected sheep, values for days 8-12 after booster vaccination represent those of sheep 662 only, while those of days 6-12 in the uninfected sheep were from sheep 569 only.

presented as mean values of two infected/vaccinated and two uninfected/vaccinated sheep.

6.3.1 Parasite Kinetics

Trypanosomes were detectable in the lymph 12 days after the infection. Two peaks of parasitosis were observed. The first minor peak occurred between days 22 to 24 p.i. when up to 2.7×10^6 parasites ml^{-1} were counted. A second, much higher trypanosome count was observed between days 82 to 86 p.i., when up to 6×10^7 trypanosomes ml^{-1} were counted (Figure 6.1). At other times, parasitosis was low or undetectable.

6.3.2 Lymph Output

Lymph output was slightly higher in the infected sheep prior to vaccination. Following primary vaccination, there was increase in lymph output in both groups of sheep but the output became higher in the uninfected sheep during the first 4 days post vaccination, p.v. (Figure 6.2). Thereafter, the daily lymph output became much higher in the infected group. After secondary (booster) vaccination, increase in lymph output was not as high as during the primary response in either group until day 8 post booster (p.b.) in the infected group. Challenge of the uninfected sheep 569 with 2×10^6 trypanosomes on day 7 p.b. resulted in a decrease in lymph output (Figure 6.2).

6.3.3 Cell Composition and Output

In both groups of sheep, prefemoral efferent lymph contained 95 percent lymphocytes, 2-3 percent granulocytes and 2-3 percent erythrocytes during the first 3 to 4 days following surgical cannulation of the lymphatic duct. By day 5 post surgery, the cellular composition had become 100 percent lymphocytic as assessed by the examination of Giemsa-stained cytospin smears. However, following primary vaccine inoculation, prefemoral efferent lymph contained 98.5 to 99 percent lymphocytes and 1 to 1.5 percent granulocytes on day one p.v. Subsequently, the cell composition was once again entirely lymphocytic.

The cell output in both groups of sheep is shown in Figure 6.3a. Initially, cell output was higher in the infected sheep. This increased further after primary vaccination, peaking at 18.1×10^7 cells hr^{-1} by day 8 p.v. (day 25 p.i.). The output then decreased between days 10 to 13 p.v. (days 27-30 p.i.) before beginning to rise again. Booster vaccination was not accompanied by further increases but rather, the cell output steadily declined until day 11 p.b. (day 88 p.i.) when there was again a marked increase (Figure 6.3a). In the uninfected sheep, primary vaccination was

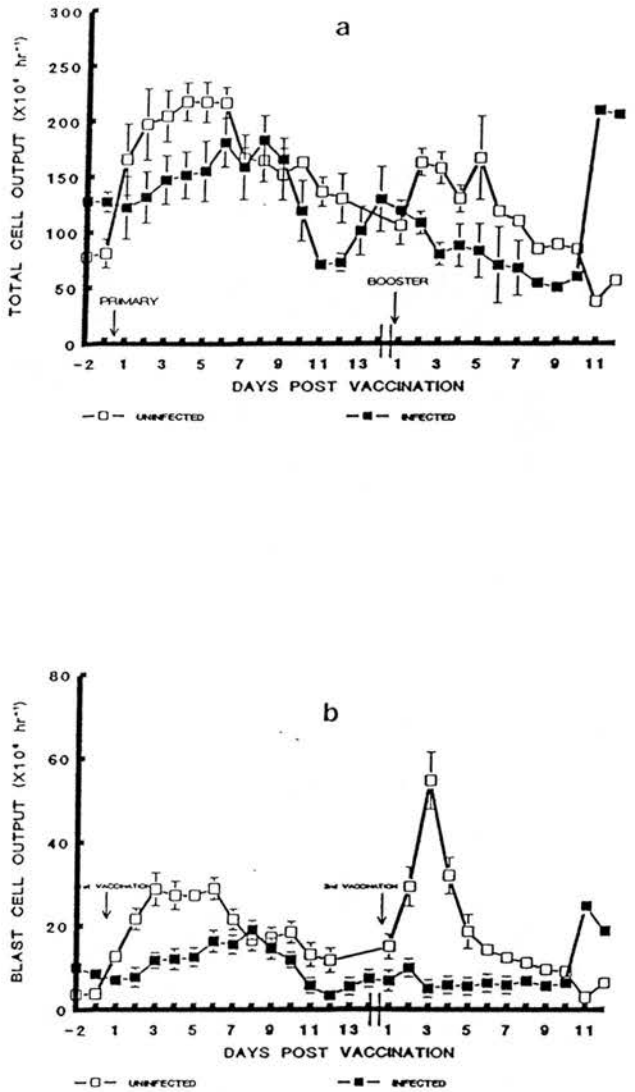


FIGURE 6.3 (a) Total cell output per hour in efferent lymph of sheep after infection with *T. evansi* and/or vaccination with *P. haemolytica* (Mean ± S.D.). Following primary vaccination, output was higher in uninfected sheep than the infected ones. A secondary response occurred in the uninfected sheep while cell output continued to decline in the infected animals even after booster vaccination. A large secondary response occurred following a decline in the second peak of parasitosis (see Figure 6.1).

(b) Hourly output of lymphoblast (Mean ± S.D.) in the same sheep. This largely mirrored the trend in cell output with the secondary response in infected sheep again occurring after a drop in the second peak of parasitosis.

In all cases, values for days 6-12 and 8-12 post booster in the uninfected and infected sheep respectively represent those of one sheep each.

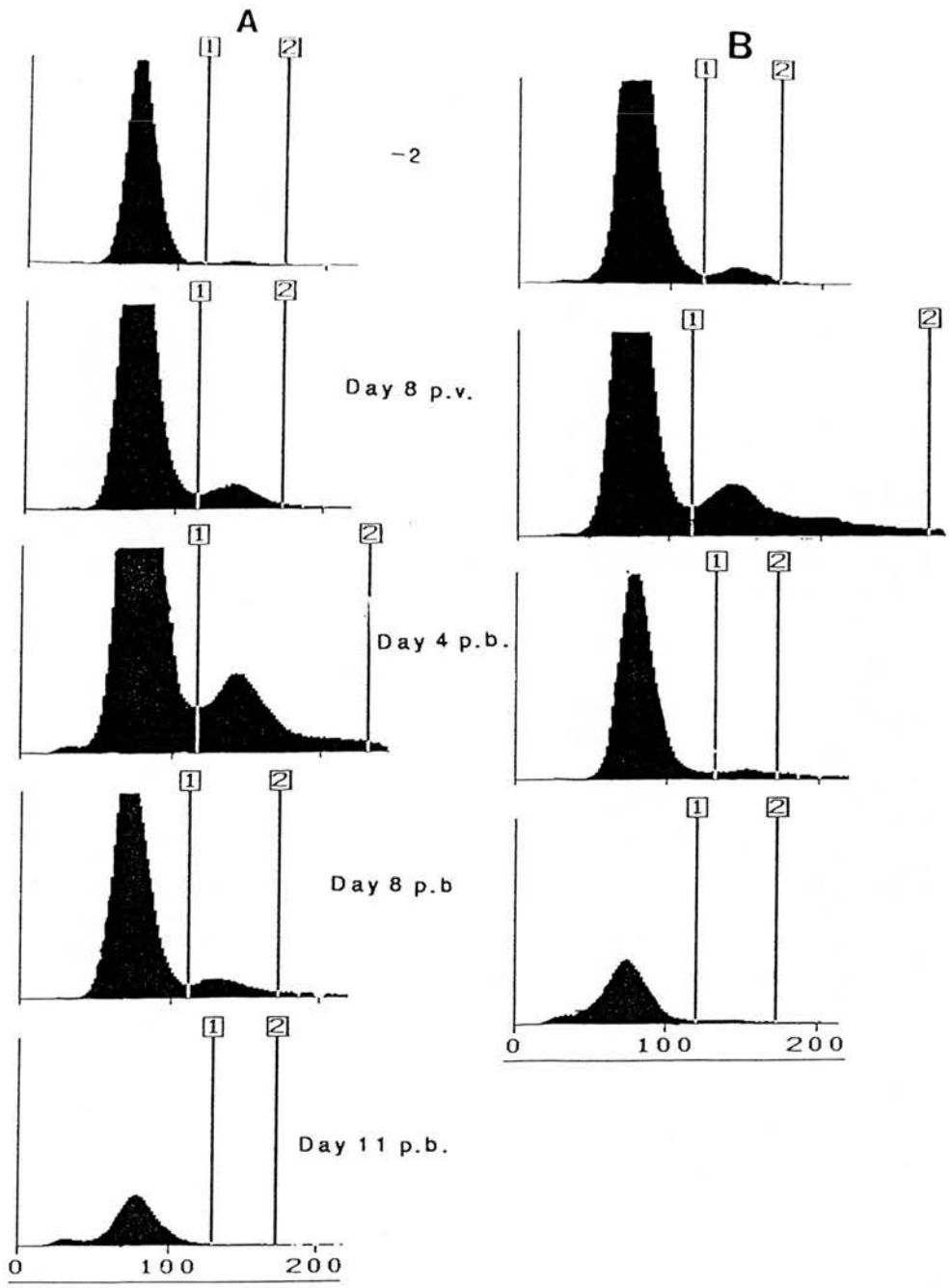


FIGURE 6.4 System 9000 histogram of efferent lymph cell count in sheep after infection with *T. evansi* TREU 2143 and/or inoculation with *Pasteurella haemolytica* vaccine. Column A = sheep 569 (uninfected/vaccinated) Column B = sheep 669 (infected/vaccinated) Gate 1 = small Lymphocyte population; Gate 2 = Lymphoblast population.
Note the decrease in the infected sheep 669 and also in sheep 569 after it was challenged with 2 x 10^6 *T. evansi* TREU 2143 (Day 11 p.b)

accompanied by a marked increase in cell output over and above what it was in the infected sheep, peaking at more than 2-fold prevaccination values on days 4 and 5 p.v. (Figure 6.3a). Thereafter, there was a gradual decline although the cell output still remained higher than the prevaccination values. Increase following secondary vaccination was generally less marked, except on day 4 post booster and challenge of sheep 569 with *T. evansi* on day 7 p.b. resulted in a sharp decrease in cell output (Figure 6.3a).

6.3.4 Output of Lymphoblasts

The infected sheep had higher numbers of lymphoblasts than the uninfected ones prior to vaccination. Following primary vaccination, there was up to a 2-fold increase which peaked by day 8 p.v. (Figure 6.3b). Thereafter and even after booster vaccination, lymphoblast numbers decreased until day 11 p.b. when there was a sharp increase. The increase in the number of lymphoblasts in the uninfected sheep following primary vaccination was quite dramatic, rising up to an 8-fold increase by day 3 p.v. (Figure 6.3b). Booster vaccination was accompanied by even higher increases of up to 14-fold prevaccination values. The challenge of sheep 569 intravenously at day 7 p.b. with 2×10^6 *T. evansi* resulted in further decrease in the output of lymphoblasts. Figure 6.4 is a System 9000 automatic cell analyser printout of frequency histograms of efferent lymph cell counts and shows increases in the cell and lymphoblast output in sheep 569 and 669.

6.3.5 Phenotypic Analysis of Efferent Lymphocytes

6.3.5.1 T Cell Phenotypes

Prior to vaccination, the number and proportion of $CD5^+$ T cells were higher in the infected group. Approximately 88 percent, which represents a mean absolute number of about 20×10^6 cells ml^{-1}, were $CD5^+$ (Table 6.2; Figure 6.5a). Following vaccination, the mean numbers of $CD5^+$ cells showed an initial decrease but this was followed by partial recovery between days 3 and 4 p.v. (20 and 21 p.i.). Thereafter, the general trend was that of a continued decline, with a marked decrease occurring 11 days p.v. Secondary vaccination was followed by a slight increase during the following week but further marked decreases corresponding with the period of the second peak of parasitosis had occurred by day 8 after booster vaccination (Figure 6.5a). The absolute numbers of $CD4^+$ as well as the numbers and proportions of $\gamma\delta$ T cells decreased generally (Figure 6.5b, d; Table 6.2). The decreases in the number of $CD4^+$ cells occurred despite the minor alterations in their proportion (Table 6.2) and were therefore a reflection of the decreases in total cell output. Following both primary and booster vaccinations, there was no marked

Table 6.2 **Proportional changes in T cell subsets in efferent lymph of sheep infected with *T. evansi* and/or inoculated with *P. haemolytica* vaccine. Figures are Mean ± S.D. of data from 2 sheep in each group except on days 6-12 and 8-12 post booster in the uninfected and infected sheep respectively when data was taken from one animal each.**

Days Post Infection	CD5		CD4		CD8		γδ T cells	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
16	75.9±6.1	88.8±0.5	67.7±2.4	55.0±6.5	13.4±2.3	26.1±2.6	6.4±0.6	6.2±0.7
17	76.2±6.4	87.4±2.9	56.4±4.8	51.3±3.5	13.1±3.0	25.0±2.5	6.4±0.7	7.1±1.0
				Primary Vaccination				
18	87.4±6.0	88.9±0.8	65.9±2.5	52.9±6.5	18.3±2.8	29.0±3.5	7.5±0.5	7.0±0.2
19	78.8±8.9	88.6±3.1	58.0±3.5	56.9±7.8	16.2±1.5	32.1±2.7	6.8±0.3	5.9±0.1
20	81.9±8.9	90.1±4.5	59.2±2.7	62.2±5.7	16.7±1.3	32.2±2.1	6.4±0.2	5.4±0.1
21	81.8±8.9	87.8±5.8	59.1±2.4	57.4±5.1	17.3±3.0	34.2±3.6	6.0±0.1	5.1±0.3
22	76.4±7.2	81.5±6.1	57.0±3.2	52.7±2.8	16.4±2.0	31.7±0.4	6.2±0.2	3.9±0.2
23	76.3±6.3	84.1±6.2	57.4±1.2	50.2±5.6	16.0±2.5	34.4±1.8	6.4±1.0	3.3±0.4
24	76.8±8.0	81.6±0.5	55.1±8.5	48.9±2.1	14.9±1.3	33.3±3.5	6.1±0.1	3.1±0.1
25	76.2±6.0	71.2±5.3	54.5±8.1	41.1±0.4	16.5±1.5	29.3±2.8	4.9±0.3	2.6±0.1
26	78.4±5.5	75.6±2.7	56.0±4.8	46.5±3.5	16.9±2.5	31.7±2.6	5.2±1.0	3.0±0.2
27	79.7±9.0	84.2±2.1	56.9±6.1	51.0±2.7	17.7±1.7	32.7±2.8	8.3±1.1	2.8±0.2
28	74.6±7.7	85.7±0.9	57.3±4.7	51.2±5.9	13.5±1.5	35.7±2.8	4.4±0.4	3.6±0.3
29	74.5±6.8	83.3±5.1	55.8±3.5	50.5±5.1	13.8±1.4	29.9±3.5	3.1±0.9	3.1±0.7
30	-	76.9±1.0	-	47.9±2.9	-	31.0±3.9	-	3.6±0.5
31	-	74.8±1.2	-	40.2±3.4	-	26.4±1.7	-	2.5±0.3
				Secondary Vaccination				
78	79.9±6.0	79.4±4.8	58.6±4.9	48.7±6.0	15.7±1.1	32.7±1.4	4.4±0.2	4.2±0.1
79	75.8±9.9	81.8±2.9	55.4±8.2	49.1±0.3	15.2±1.4	33.1±2.2	5.0±0.2	3.4±0.1
80	77.2±6.5	87.1±1.4	53.8±3.6	59.0±4.3	15.6±1.6	33.5±1.2	5.0±1.0	3.7±0.1
81	79.7±6.2	86.7±4.5	57.5±2.6	59.3±5.1	15.9±2.0	26.5±2.4	6.2±0.6	3.7±0.7
82	74.5±5.4	68.9±6.6	53.8±8.2	40.3±3.1	14.1±1.4	24.2±0.6	5.0±0.5	3.5±0.5
83	67.9±0.0	78.2±0.4	40.8±0.0	52.8±3.5	11.6±0.0	26.7±3.0	9.3±0.0	2.8±0.8
84	77.5±0.0	80.4±2.9	41.5±0.0	55.2±5.0	11.4±0.0	27.1±1.5	14.7±0.0	2.9±0.4
85	75.6±0.0	76.7±0.0	34.3±0.0	51.1±0.0	11.4±0.0	26.8±0.0	8.0±0.0	3.4±0.0
86	59.9±0.0	76.9±0.0	34.5±0.0	51.4±0.0	9.4±0.0	28.9±0.0	6.5±0.0	4.2±0.0
87	73.6±0.0	77.4±0.0	40.4±0.0	46.8±0.0	11.7±0.0	29.1±0.0	7.1±0.0	3.0±0.0
88	85.8±0.0	79.1±0.0	55.5±0.0	46.6±0.0	11.3±0.0	35.7±0.0	11.6±0.0	2.7±0.0
89	88.1±0.0	68.6±0.0	66.3±0.0	49.5±0.0	13.1±0.0	24.2±0.0	2.6±0.0	2.7±0.0

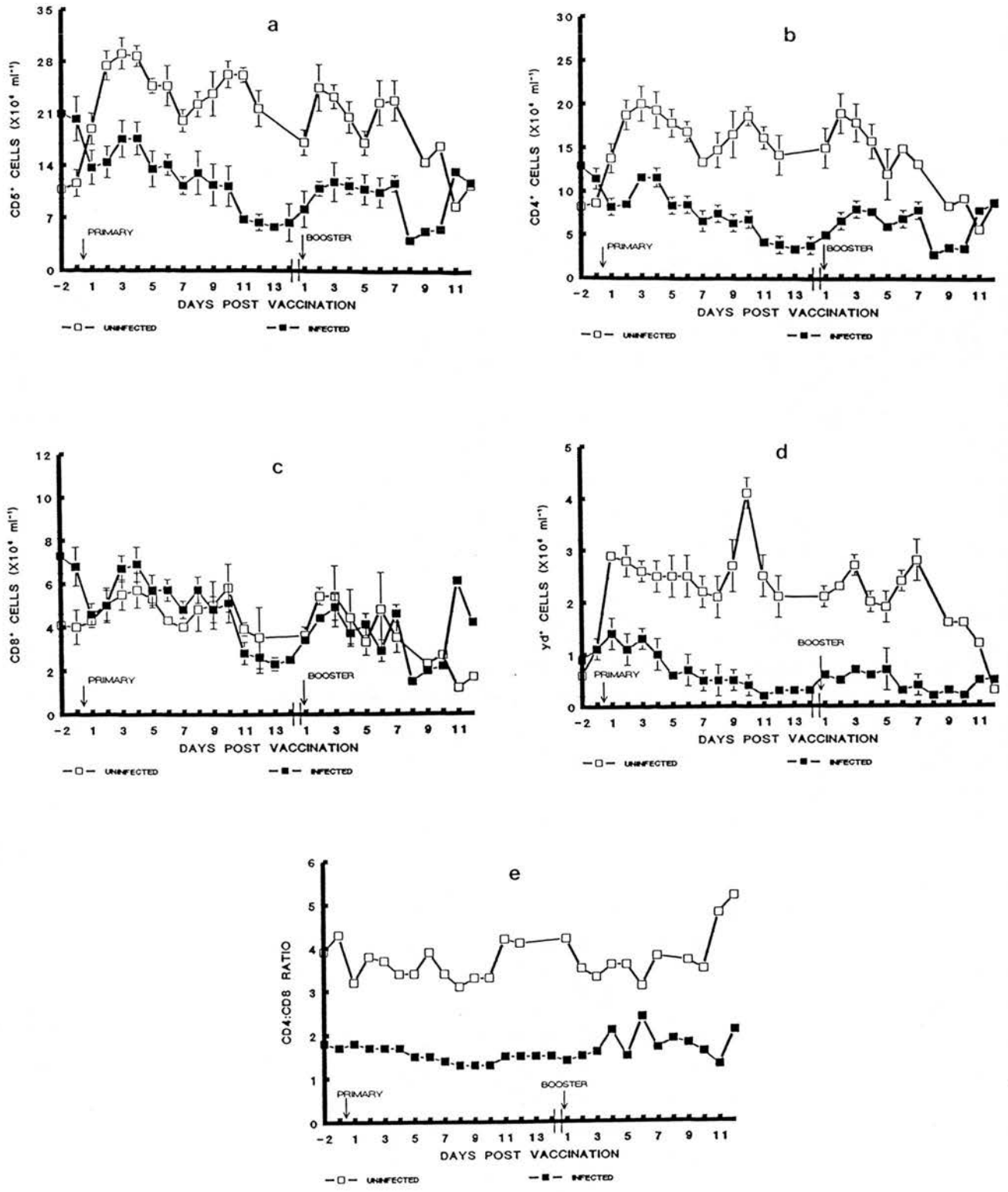


FIGURE 6.5 T cell subset responses in sheep infected with *T. evansi* and/or inoculated with *P. haemolytica* vaccines. Values represent Mean ± S.D. of two animals in each case except for days 6-12 and 8-12 post booster in the uninfected and infected sheep respectively when they represent values derived from one animal each.

variation in the absolute number of $CD8^+$ T cells until day 11 p.v. and 8 p.b. when marked decreases were also observed (Figure 6.5c). This was because of the increases observed in the percentages during those periods (Table 6.2). Thus, throughout the period of the experiments, the CD4:CD8 ratio decreased and remained below 2 at most times (Figure 6.5e).

In the uninfected animals about 76 percent of prefemoral efferent lymphocytes (Table 6.2), representing an approximate mean number of 11 x 10^6 cells ml^{-1} were $CD5^+$ before priming with *Pasteurella* vaccine (Figure 6.5a). Both primary and secondary vaccine administration was accompanied by large increases in the number of efferent lymphocytes expressing the T cell associated antigens CD5, CD4, CD8 and γδ surface molecules (Figure 6.5a, b, c and d), even though their percentages remained largely unchanged or showed only marginal increases (Table 6.2). Except for $CD8^+$ T cells where the numbers were similar to those of the infected group, the numbers of $CD5^+$, $CD4^+$ and γδ T cells were more than twice their corresponding values in the infected group at all time points which again was a reflection of the large increases in total cell output. Moreover, the CD4:CD8 ratio in this group was over two times greater than in the infected group (Figure 6.5e). However, following challenge of sheep 569 with *T. evansi* on day 7 p.b., all T cell subsets showed a decrease in their numbers and proportions.

6.3.5.2 B Cell Kinetics

Following *T. evansi* infection and/or inoculation with *Pasteurella* vaccine in sheep, the kinetics of efferent B cells were monitored using VPM 30 (anti-sheep B cell) and SBU-T6 (anti-sheep CD1 which recognises B cells as well as cortical thymocytes and Langerhans cells). In the uninfected sheep, primary and secondary vaccinations were accompanied by marked increases of up to 3-5-fold prevaccination values in the number of circulating B cells (Figure 6.6a, b). This is as a result of the increases observed in their proportions (Table 6.3) and also of the large increases in the cell output following the vaccinations.

In contrast, in infected sheep, although marked increases occurred in the proportions of $CD1^+$ and VPM 30^+ cells after both primary and booster vaccinations (Table 6.3) there was little variation in their absolute numbers until after days 10 and 7 post-primary and booster vaccinations respectively when there was a marked decrease as a result of decrease in total cell output (Figure 6.6a, b). Moreover, the output of B cells was much less in these animals in comparison to the output in the uninfected control sheep.

Table 6.3 **B cell kinetics as shown by the percentages of VPM 30$^+$, CD1$^+$ and MHC Class II$^+$ cells in efferent lymph of sheep infected with *T. evansi* and/or inoculated with *P. haemolytica* vaccine. Figures represent Mean ± S.D. of two sheep in each case except during days 6-12 and 8-12 post booster in the uninfected and infected sheep respectively when figures were taken from only one sheep each.**

Days Post vaccination	VPM 30		CD1		MHC Class II	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
-2	19.2±0.4	10.9±1.3	8.0±1.4	9.1±1.5	23.8±2.5	26.8±1.7
-1	20.5±2.7	13.1±1.0	8.7±1.1	10.5±0.6	25.3±3.1	25.6±1.8
			Primary Vaccination			
1	23.7±2.9	15.0±2.6	12.3±0.6	9.7±0.3	26.0±0.5	16.0±1.5
2	21.6±1.8	12.6±1.5	16.8±2.1	12.0±1.8	27.8±2.3	18.1±1.2
3	30.4±3.2	14.2±1.8	17.3±1.8	14.9±1.7	29.6±2.8	22.2±1.8
4	26.0±1.2	17.9±2.9	16.2±1.9	13.2±1.4	26.5±1.6	23.5±1.7
5	33.9±0.5	19.1±2.0	15.6±2.2	23.4±0.7	32.5±3.5	38.3±1.2
6	31.4±1.6	16.4±1.6	20.0±3.0	17.1±1.2	33.4±2.0	29.2±2.7
7	37.3±2.3	17.6±1.1	19.4±2.6	25.5±1.3	32.4±2.5	35.1±1.1
8	33.6±1.8	20.9±2.0	24.7±3.1	27.3±1.7	33.3±4.1	40.0±2.1
9	29.3±0.7	21.5±1.9	21.9±1.7	22.4±1.4	32.4±2.6	42.2±2.5
10	26.2±1.9	14.5±0.7	17.7±1.8	17.4±1.9	31.9±3.8	31.2±2.5
11	29.2±0.8	17.8±1.5	13.4±1.6	17.9±1.9	34.1±4.0	30.1±1.0
12	35.3±0.8	15.7±1.4	13.7±1.6	20.2±2.0	31.4±3.9	28.7±1.9
13	-	19.4±1.2	-	31.6±0.6	-	42.1±1.0
14	-	15.8±0.6	-	27.5±1.4	-	32.9±2.2
			Secondary Vaccination			
1	34.1±2.1	18.2±1.9	11.9±0.7	31.4±1.6	31.4±3.6	43.3±2.9
2	31.9±3.0	18.6±1.4	18.0±1.9	21.4±1.9	31.7±4.2	37.9±3.2
3	47.2±5.1	18.5±1.2	6.1±0.4	28.3±2.2	33.8±2.7	30.9±1.8
4	37.9±2.1	16.8±0.9	9.1±0.8	25.3±1.0	34.8±2.6	26.5±2.4
5	36.6±2.6	22.6±0.3	19.6±0.4	31.2±1.7	36.5±2.1	32.8±0.7
6	39.3±0.0	23.5±2.4	24.4±0.0	30.2±2.9	53.6±0.0	45.4±2.0
7	53.9±0.0	19.8±1.8	ND	22.0±1.6	51.9±0.0	44.4±2.5
8	40.0±0.0	12.8±0.0	ND	27.6±0.0	54.0±0.0	18.0±0.0
9	39.9±0.0	11.8±0.0	ND	33.5±0.0	60.4±0.0	12.4±0.0
10	42.6±0.0	11.6±0.0	ND	28.4±0.0	55.9±0.0	17.9±0.0
11	43.1±0.0	10.6±0.0	ND	21.9±0.0	59.8±0.0	11.3±0.0
12	22.3±0.0	23.2±0.0	ND	30.8±0.0	50.8±0.0	11.0±0.0

ND = Not determined

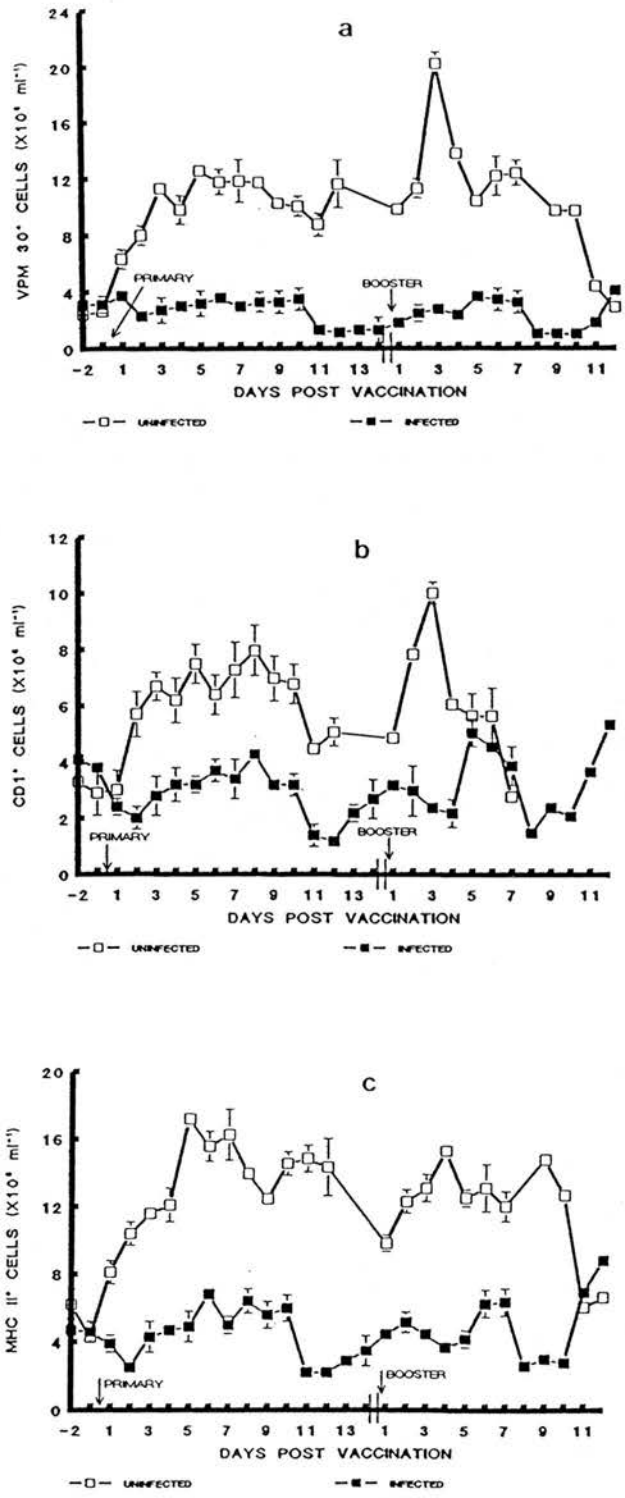


FIGURE 6.6 B cell kinetics in efferent lymph of sheep infected with *T. evansi* and/or inoculated with *P. haemolytica* vaccine. Figures represent Mean absolute cell counts per ml ± S.D. Infection resulted in a depressed B cell response to the vaccine antigen being much less in the infected sheep.

Table 6.4 **Mean percent of (a) $CD4^+$ cells coexpressing the CD8 antigen, (b) $CD8^+$ cells coexpressing the CD4 antigen and (c) $CD5^+$ B cells in efferent lymph of sheep infected with *T. evansi* and/or inoculated with *P. haemolytica* vaccine.**

	(a)		(b)		(c)	
Post vaccination (days)	Mean % $CD4^+$ for CD8 Uninfected	Infected	Mean % $CD8^+$ for CD4 Uninfected	Infected	Mean $CD5^+$ B cells (%) Uninfected	Infected
-2	4.6	9.6	12.9	30.8	18.7	53.3
-1	4.2	11.8	10.6	33.3	12.9	54.0
			Primary vaccination			
1	5.8	15.1	14.7	35.1	18.7	60.6
2	5.9	16.6	16.9	42.2	14.6	70.0
3	5.9	18.3	17.8	56.7	13.6	67.4
4	4.8	21.1	15.9	55.1	17.8	74.2
5	5.1	17.4	15.8	42.2	14.2	56.7
6	4.6	13.4	13.8	31.4	14.3	66.3
7	6.2	13.9	12.2	28.5	14.1	62.6
8	5.7	17.5	13.5	19.0	14.8	58.2
9	5.1	18.0	11.8	20.0	12.1	59.0
10	5.4	19.8	12.2	20.5	11.7	65.3
11	4.3	15.6	12.1	27.5	14.5	69.4
12	4.2	20.3	11.3	30.3	14.1	60.2
13		15.2		21.8		58.3
14		12.7		17.3		34.4
			Secondary vaccination			
1	6.5	12.5	12.8	26.9	17.5	47.8
2	7.8	14.1	16.7	27.8	16.9	50.7
3	5.7	20.5	13.6	31.2	16.9	59.4
4	4.8	14.5	13.0	32.6	10.0	49.4
5	6.2	8.4	11.6	26.9	17.2	71.9
6	5.9	13.8	10.7	28.7	10.9	67.6
7	5.2	13.5	15.1	33.9	15.2	75.2
8		11.0		30.1		61.1
9	9.6	15.4	19.1	37.3	15.6	74.2
10	6.2	17.3	11.4	31.7	17.3	84.6
11	16.9	24.0	13.2	57.3	19.4	76.2
12	13.6	21.3	18.7	45.9	10.4	70.3

6.3.5.3 Coexpression of CD4 and CD8 by Efferent Lymphocytes

Two colour immunofluorescence staining using the anti-CD8 mAb, S-T8 and the biotinylated anti-CD4 mAb, SBU-T4 and flow cytometry revealed one of the most surprising and interesting finding of these studies. It was observed that *T. evansi* infection induced the coexpression of the CD4 and CD8 molecules by efferent lymphocytes, which was made more obvious by stimulation of the lymph node with *Pasteurella haemolytica* vaccine. For instance, following primary and booster vaccination, there were further increases in the proportion of $CD4^+$ population coexpressing the CD8 molecule and vice versa (Table 6.4)

In contrast, in the uninfected group, few $CD4^+CD8^+$ (double positive) T cells were observed prior to or after primary and secondary vaccinations. The proportion of $CD4^+$ cell population bearing the CD8 antigen at no time exceeded 6 percent nor did that of $CD8^+$ cells bearing the CD4 antigen exceed 15 percent at any time point. Figure 6.7 shows FACS contour profiles of representative animals (sheep 569, uninfected and 669, infected) illustrating these increases in $CD4^+CD8^+$ double positive efferent lymphocytes. The percentages of $CD4^+$ cell population bearing the CD8 antigen and vice versa in the two groups of sheep at various time points are shown in Table 6.4.

6.3.5.4 Expression of CD5 Antigen on Efferent B Cells

In common with the finding in peripheral blood, *T. evansi* induced increased expression and output of $CD5^+$ B cells by prefemoral efferent lymphocytes. Prior to vaccination and at about 17 days p.i., up to 50 percent of B cells bore the CD5 antigen in the infected sheep. This was further increased by primary vaccination when an average of 60 to 74 percent of B cells were $CD5^+$. Following booster vaccine administration, the proportions and numbers of $CD5^+$ B cells remained high with an average of between 40 to 85 percent (Table 6.4). In contrast, the uninfected group showed no marked difference between the pre- and post-vaccination levels of $CD5^+$ B cells remaining between 10 and 20 percent throughout the experiment (Table 6.4). Figure 6.8 are FACS contour profiles illustrating $CD5^+$ B cell expression in infected and uninfected animals.

6.3.5.5 Expression of MHC Class I, CD45 and MHC Class II Antigens

In both groups, the proportion of cells expressing the MHC Class I and CD45 antigens exceeded 98 percent at all time points. Their absolute numbers therefore, mirrored the increases or decreases in total cell output. There were marked increases in MHC Class II expression following vaccination in the infected

FIGURE 6.7 Representative FACS contour profiles of sheep 569 (uninfected/vaccinated) and sheep 669 (infected/vaccinated) illustrating differences in the induction of $CD4^+CD8^+$ T cell population in efferent lymph after infection with *T evansi* TREU 2143 and/or immunisation with *Pasteurella haemolytica* vaccine

Panel A = sheep 569; Panel B = sheep 669

In each of the profiles :

Quadrant 1 = $CD4^+$ cell population

Quadrant 2 = $CD4^+CD8^+$ cell population

Quadrant 3 = $CD4^-CD8^-$ cell population

Quadrant 4 = $CD8^+$ cell population

Note that challenge of sheep 569 on day 7 p.b. with *T evansi* TREU 2143 was followed by the appearance of double positive cells (Panel A , day 8 and 10 p.b.) and that in sheep 669, the terminal decrease in double positive cells was accompanied by decreasing $CD4^+$ and $CD8^+$ (single positive) cell population (Panel B, day 8 and 10 p.b.)

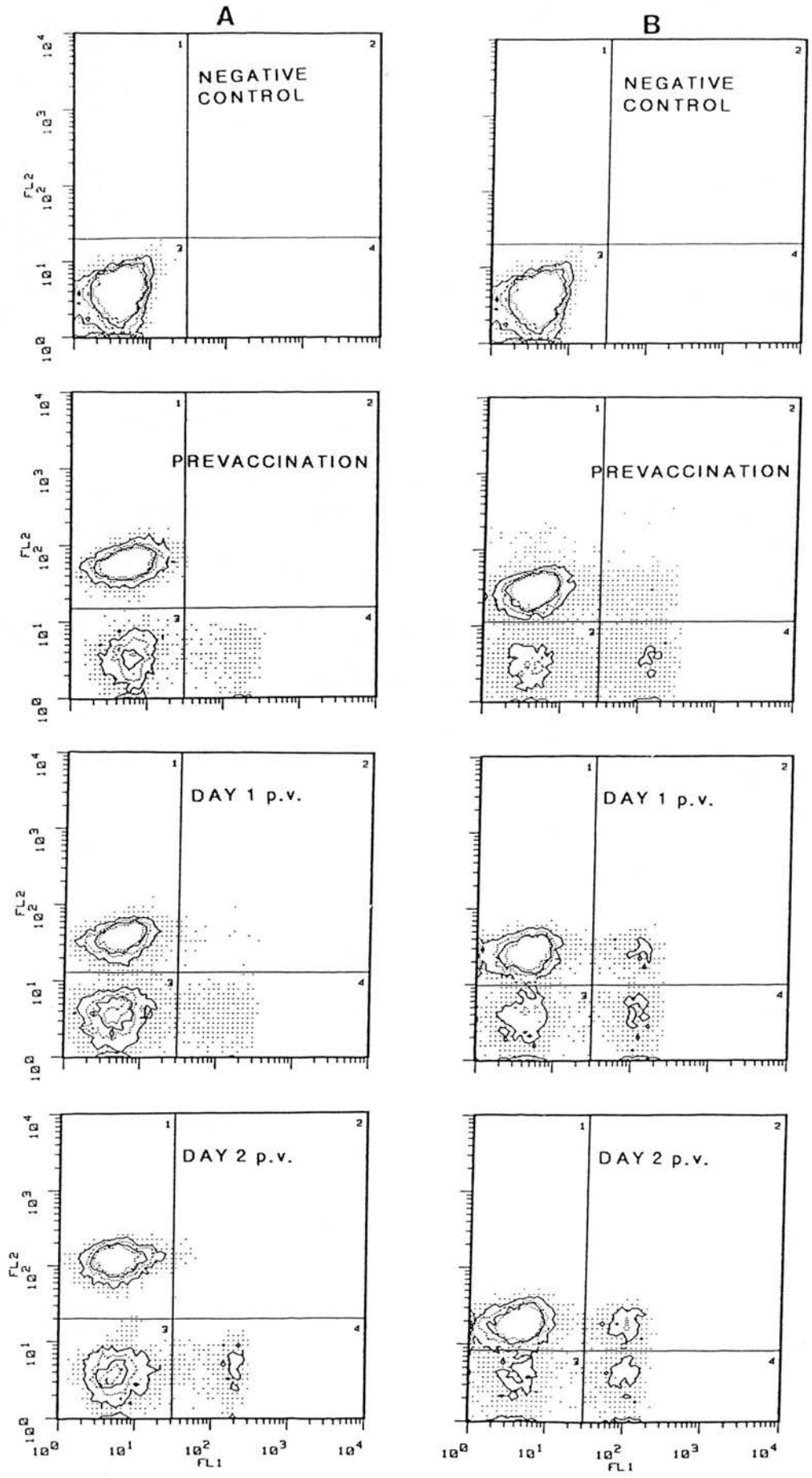

A
B
NEGATIVE CONTROL
NEGATIVE CONTROL
PREVACCINATION
PREVACCINATION
DAY 1 p.v.
DAY 1 p.v.
DAY 2 p.v.
DAY 2 p.v.
FL2
FL1
1
2
3
4

FIGURE 6.7

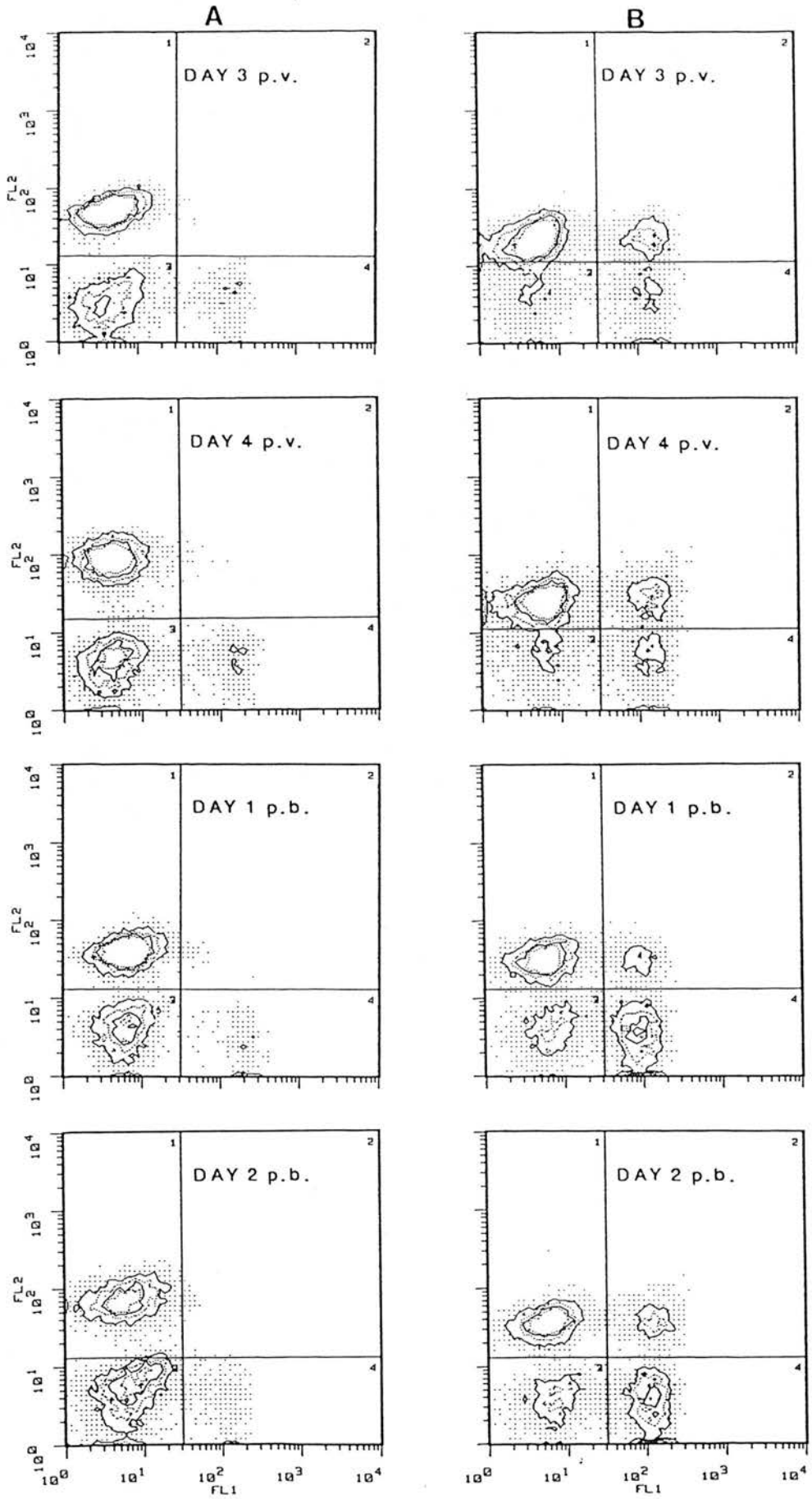


FIGURE 6.7

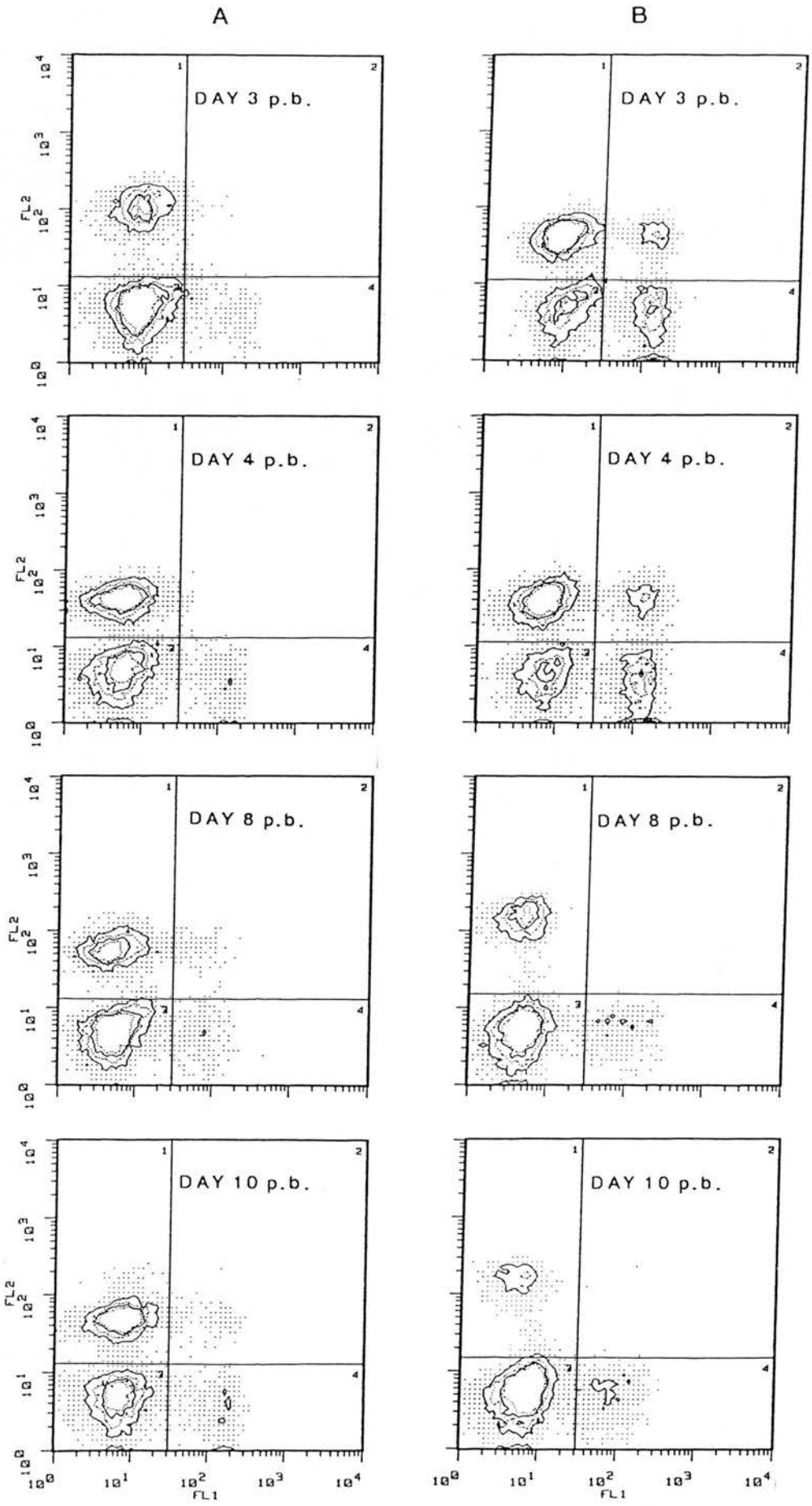


FIGURE 6.7

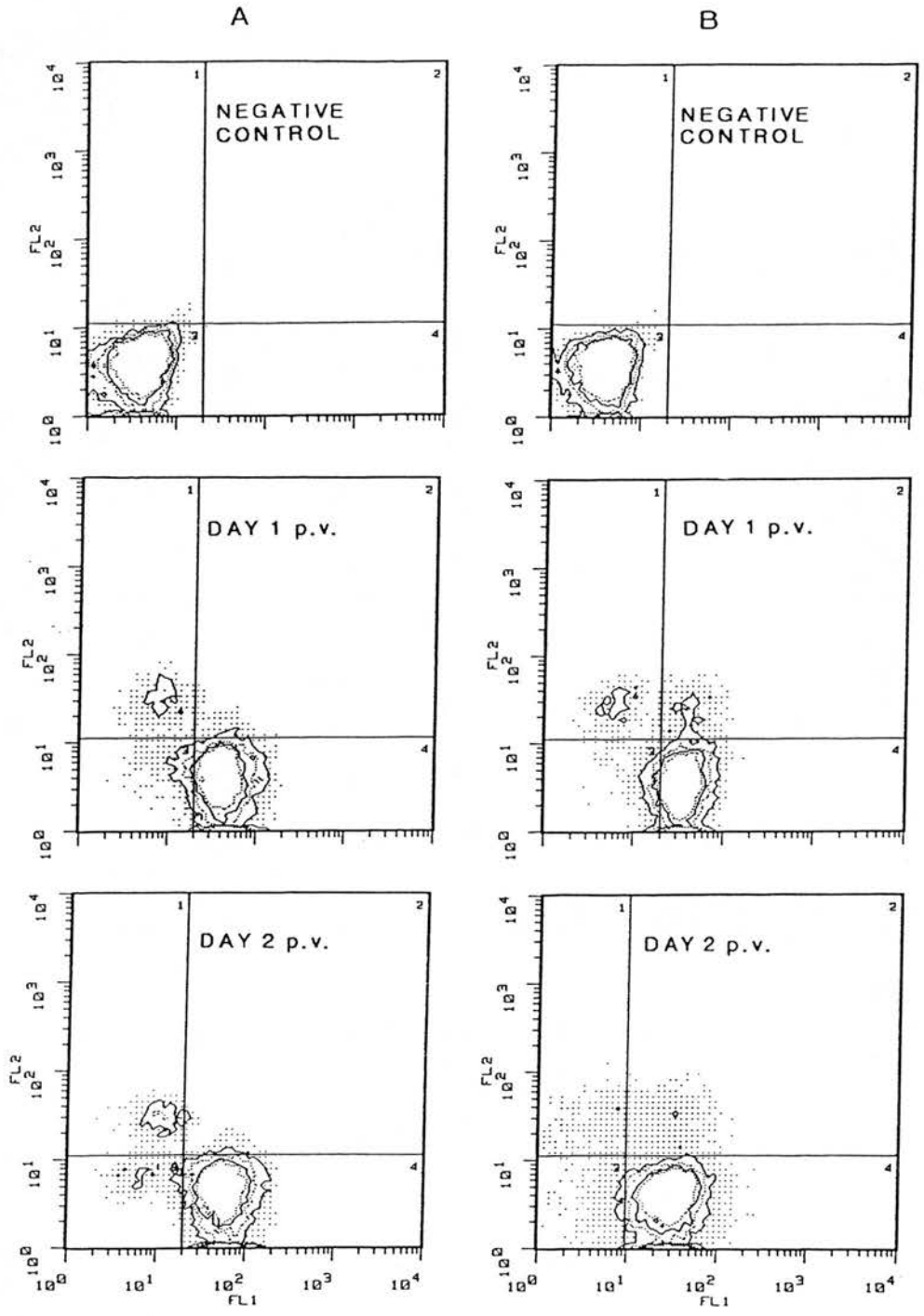


FIGURE 6.8 FACS contour profiles illustrating $CD5^+$ B cell population in efferent lymph of sheep 569 (uninfected/vaccinated) and sheep 669 (infected/vaccinated).Panel A = sheep 569 and Panel B = sheep 669. Quadrant 2 = $CD5^+$ cell population.

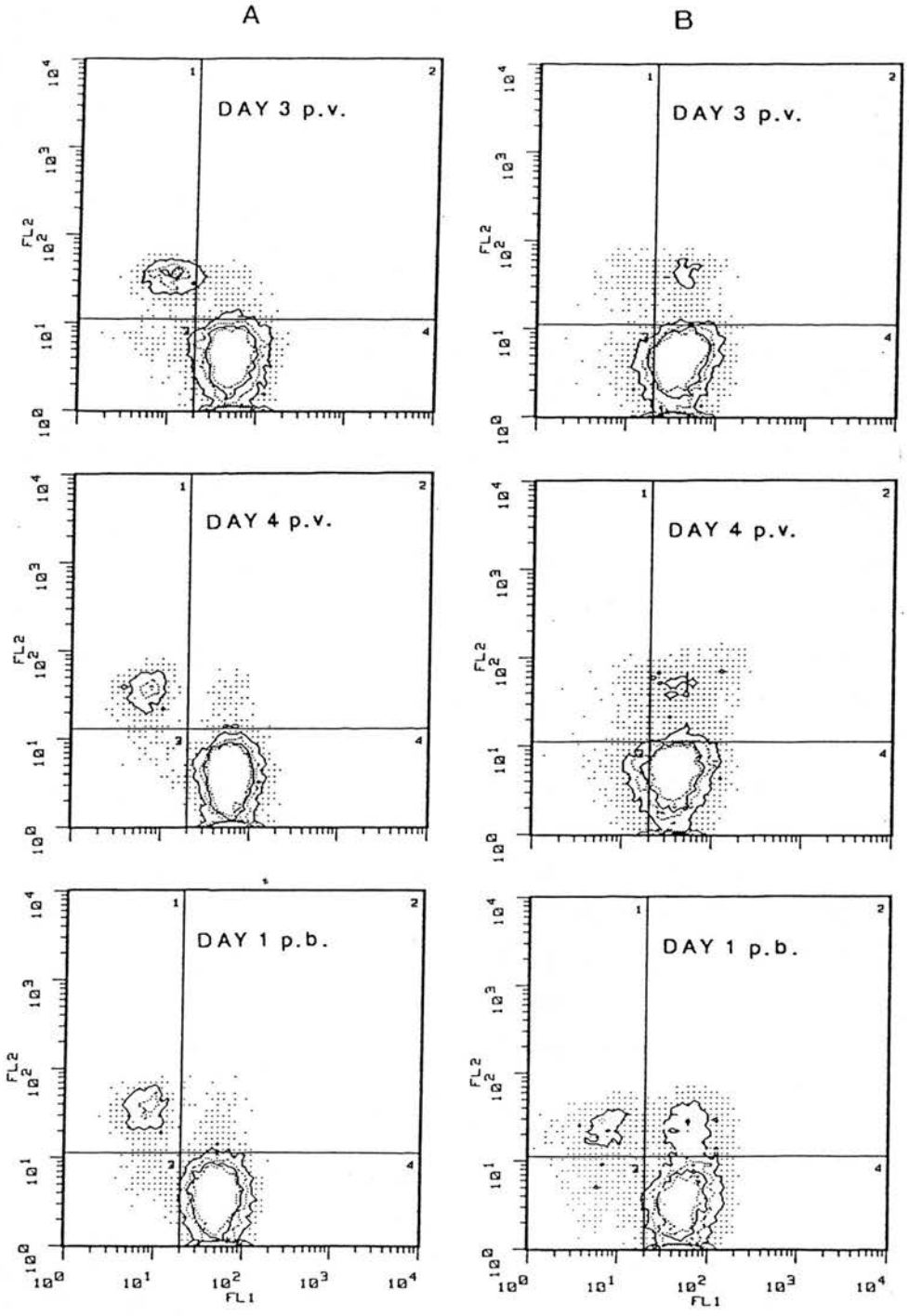


FIGURE 6.8 FACS contour profiles illustrating $CD5^+$ B cell population in efferent lymph of sheep 569 (uninfected/vaccinated) and sheep 669 (infected/vaccinated).Panel A = sheep 569 and Panel B = sheep 669. Quadrant 2 = $CD5^+$ cell population.

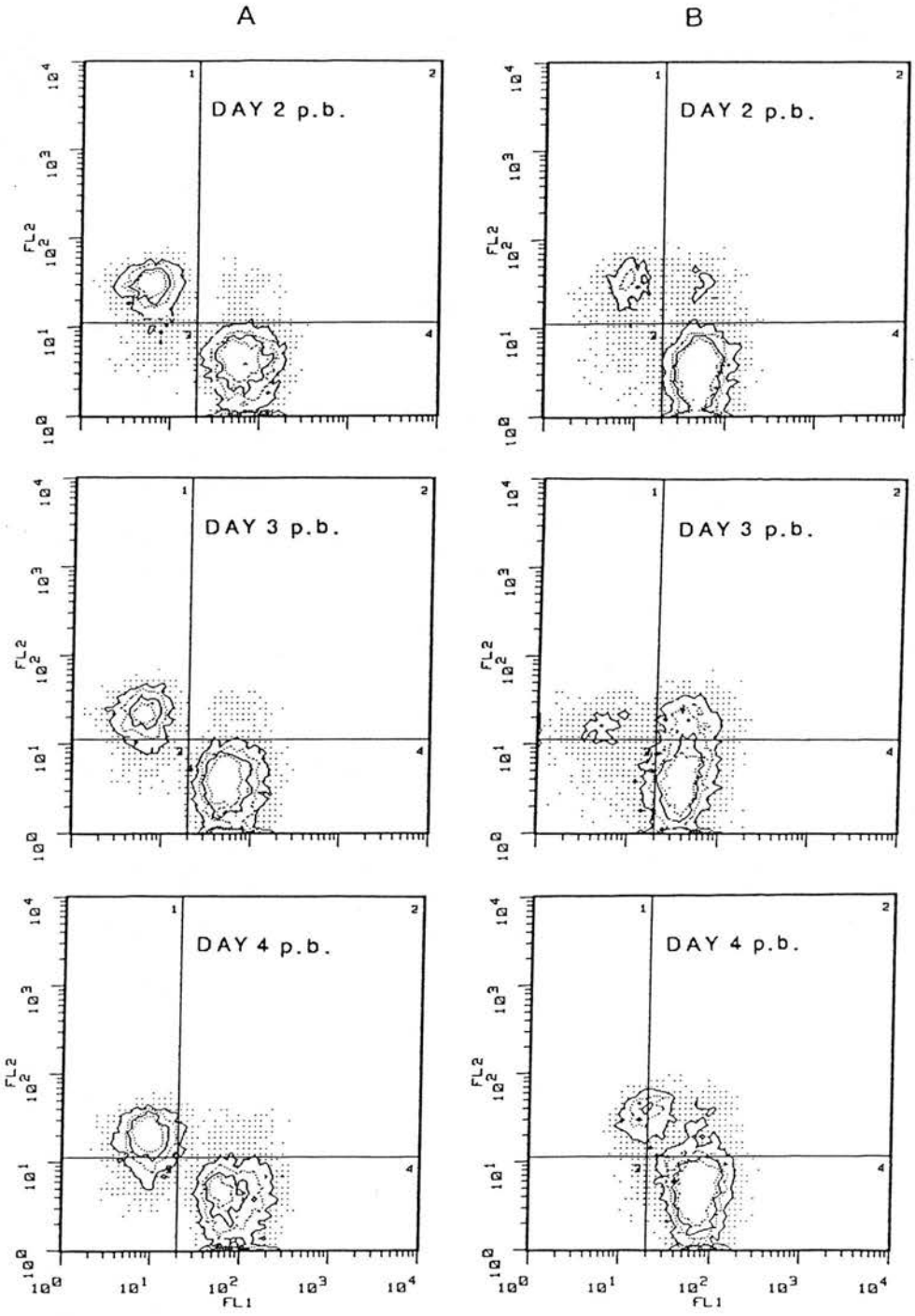


FIGURE 6.8 FACS contour profiles illustrating $CD5^+$ B cell population in efferent lymph of sheep 569 (uninfected/vaccinated) and sheep 669 (infected/vaccinated).Panel A = sheep 569 and Panel B = sheep 669. Quadrant 2 = $CD5^+$ cell population.

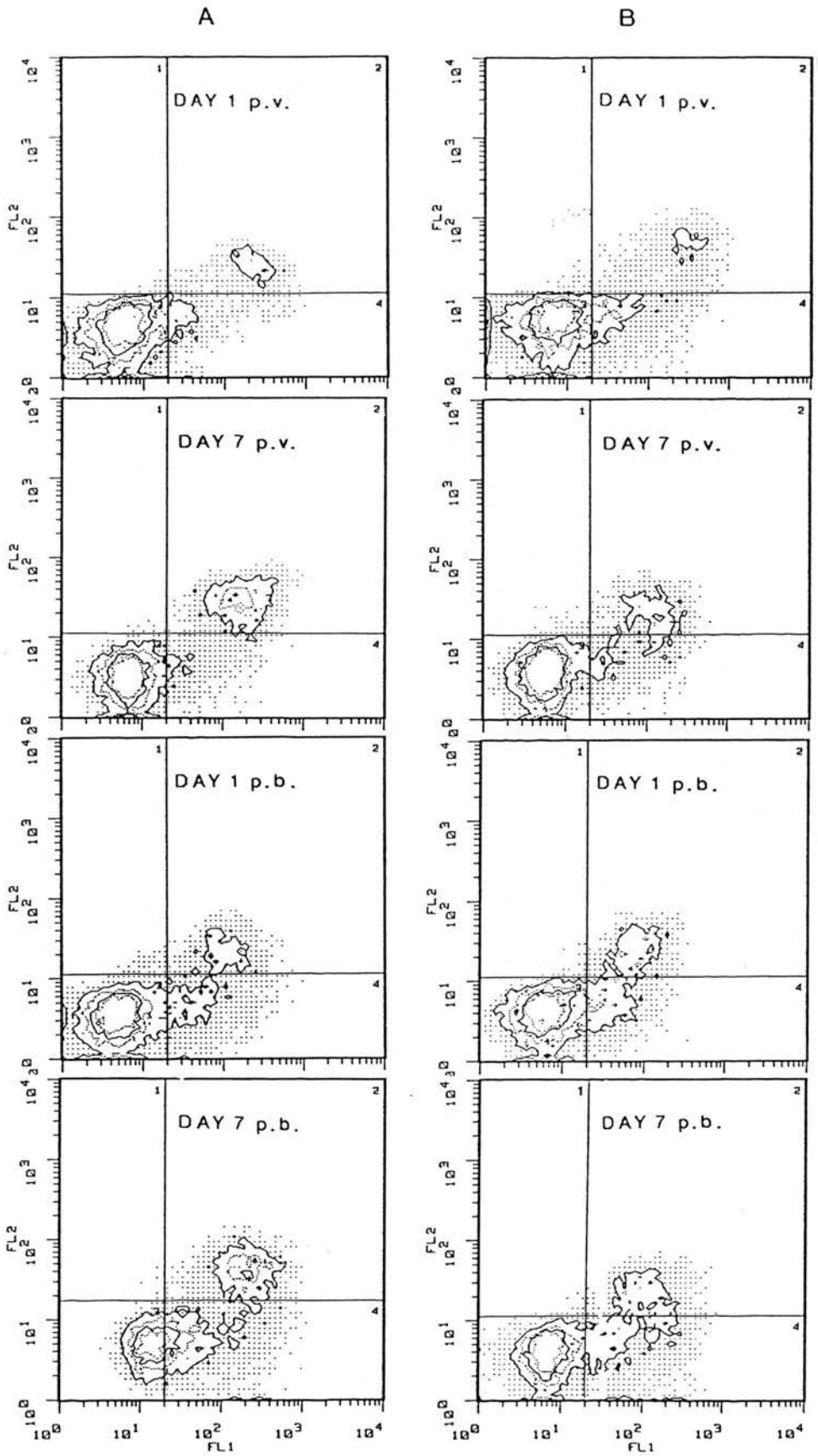


FIGURE 6.9 Contour profiles of a two-colour staining for B cell and MHC Class II expression. In both infected and uninfected sheep all B cells expressed the Class II antigen at all time points.

group (Table 6.3), although this represented only occasional marginal increases in the absolute number of cells expressing the antigen (Figure 6.6c). Dual colour flow cytometry revealed that at all times, all B cells expressed the MHC Class II antigen (Figure 6.9). In the uninfected sheep, there was more than a 4-fold increase in the number of cells expressing the MHC Class II antigen following primary and secondary vaccinations (Figure 6.6c). Similar to observations in the infected sheep, all B cells expressed Class II antigen as shown by double colour immunofluorescence (Figure 6.9).

6.4 Discussion

The intravenous infection of sheep with *T. evansi* resulted in the appearance of trypanosomes in the efferent lymph at higher numbers, and for periods longer than was observed in the peripheral blood (Chapters 4 and 5). This observation, tends to reaffirm the proposition that trypanosomes of the *brucei* group are 'humoral' since they have greater tropism for tissues and body fluids other than the blood (Losos and Ikede, 1972). For instance, *T. brucei* has the capacity to leave the blood circulation, invade tissues and enter central lymphatics in large numbers (Losos and Ikede, 1972), although similar observation has been made in goats infected with *T. vivax* (Emery, Barry and Moloo, 1980) and sheep infected with *T. congolense* (Mwangi, 1991). The relatively greater number of trypanosomes in the lymph than blood, despite the fact of the intravenous route of infection, also supports suggestions that the lymphatic system is the major route for trypanosome dissemination and that the lymph node constitutes a secondary locus for the proliferation of the parasites (Luckins and Gray, 1979; Gray and Luckins, 1980; Dwinger *et al.*, 1990). Thus, the parasites were not only able to persist in the efferent lymph, but also produced a much higher secondary level of parasitosis almost three months after the initial infection. However, this higher level of secondary parasitosis may also be a reflection of a depression in the immune competence of the sheep at this stage of the infection.

Infection of sheep with *T. evansi* affected the response of the draining lymph node to *Pasteurella* vaccine administration in terms of cell output and phenotypic composition. By comparing prevaccination figures for the infected and uninfected sheep, *T. evansi* induced a much higher cellular output. However, after vaccination, although infected animals showed further increases in cell output during the following 9 days, the increase was actually lower than in the uninfected group. In addition, as the infection progressed, so did the decrease in cell output in the infected animals. It has been shown that the cellular responses taking place within the lymph node draining a site of antigen deposition is reflected by marked

increase in the cellular output from the draining efferent lymphatic duct (Hall and Morris, 1962; 1965a, b). For instance, there is increase in cell output from the popliteal efferent lymphatic duct following subcutaneous inoculation of sheep with *Toxoplasma gondii* (McColgan *et al.*, 1987) or with *C. psittaci* (Huang *et al.*, 1991). In addition, intradermal infection of sheep with metacyclic *T. congolense* resulted in increased cell output from the prefemoral efferent duct draining from the local lymph node (Mwangi, 1991). This increase is bimodal with the initial response coinciding with the onset of parasitosis and as such differed from the observed increases with *T. gondii* and *C. psittaci* in which there was one peak. These studies are similar to observations made in the present study. Uninfected animals showed one peak each, in cellular output, after primary and secondary vaccinations. The peak following the secondary *Pasteurella* vaccination occurred after a 24 hr dip in the total cell output. This is similar to observations by Hall and Morris (1965b) and Hopkins *et al.* (1981b) that there is marked reduction in efferent lymph cell output during the first 6-20 hrs after secondary challenge of the node with antigen even though lymph flow is unaltered. This phenomenon, known as cell shutdown (Hall and Morris, 1965b), is followed by increased output of cells which, it has been suggested, comprises lymphocytes which entered the node during cell shutdown and those generated within the node in response to the antigen (Hay and Hobbs, 1977). On the other hand, the kinetic changes in cell output following vaccination was altered by prior infection with *T. evansi*. First, the overall increase was not only less than in the uninfected sheep but was gradual and peaked 4 days later than in the uninfected animals, declining rapidly thereafter, before beginning to rise to a minor second peak. Secondly, following a booster vaccination, there was hardly any increase, the cell output continuing to drop until 10 days later when it began to rise again. It seems that the combined stimulus provided by the trypanosomes and the *Pasteurella* vaccine either results in cell depletion, a prolonged cell shutdown or in the release of factors which inhibit lymphocyte migration from the node. Whichever is the case, it is also apparent that for this to occur trypanosomes must be present, since subsequent challenge of the previously vaccinated/uninfected sheep with *T. evansi* resulted in immediate and marked decrease in cell output. Trypanosome infection activates macrophages (Askonas, 1985) and it is possible that increased PGE_2 production by the activated macrophages may act to inhibit lymphocyte migration from the node. PGE_2 is not only an inhibitor of lymphocyte proliferation, but also a potent suppressor of lymphocyte migration even at very low concentrations (McCarty and Goetzl, 1979; Hopkins *et al.*, 1981a; Van Epps, 1981). Perhaps the hyperplastic nature of peripheral lymph nodes of trypanosome infected animals may result from such lymphocyte migration inhibition occasioned by PGE_2

production. A similar situation has been observed in tumour conditions. For instance, it has been observed that tumours in laboratory animals produce increased quantities of prostaglandins (Pelus and Bockman, 1979) and this has been used to explain why lymph nodes draining sites of tumour lesions are not only hyperplastic (Alexander *et al.*, 1969), but contain cells which are incapable of reacting to either antigen or mitogens (Mavligit *et al.*, 1974). Surgical removal of the main tumour mass and therefore, decrease in PG production, not only results in the mass exit of lymphocytes and blast cells from the node in question, but also in subsequent immunity to tumour challenge (Alexander *et al.*, 1969; Hopkins *et al.*, 1981b).

The stimulation of a lymph node by i.d., s.c. or i.m. injection of antigen also results in increased output of lymphoblasts and plasmablasts in the efferent lymph of the node peaking between 3 to 14 days of the antigen administration (Hopkins *et al.*, 1981a, b; McColgan *et al.*, 1987; Huang *et al.*, 1991; Mwangi, 1991; Yirrell *et al.*, 1991). The result obtained with the uninfected sheep following primary and secondary vaccination is in agreement with these reports. However, although increases in output of lymphoblasts occurred also in the infected animals, it was limited, showing marked increase only at day 11 post booster (88 days p.i.) after the decline in the second peak of parasitosis. It is of interest to note that the increase in output of lymphoblasts in the infected sheep prior to vaccination was in the order observed by Mwangi (1991) in sheep infected with *T. congolense* and that following primary vaccination there was even a greater increase before the decline by day 11 p.v. Thus, without the benefit of observing uninfected animals, the conclusion would have been that the infection had no depressive effect on lymphoblast response to the antigen. The reduced output of lymphoblasts in the infected sheep to the vaccine antigen could be due to a number of reasons. Since the subsequent challenge of the uninfected/vaccinated sheep also results in rapid reduction in blast output, it is posible that hyperactivation of APCs by the parasite and *Pasteurella* antigens induces them to increased production of PGE_2 which may then act in one of two ways. First, it may act through its suppressive action on lymphocyte effector function to limit *Pasteurella*-specific T cell proliferation and thus blast production in the node. It has been shown in sheep that efferent lymph of a node stimulated with either BCG and Johne's disease vaccine or Keyhole Limpet Haemocyanin (KLH), non-specifically suppresses the proliferation of normal PBLs when stimulated *in vitro* with a variety of antigens and that the suppressor factor is PGE_2 (Hopkins *et al.*, 1981b). Secondly, PGE_2 which mediates cell shutdown (Hopkins *et al.*, 1981a) could by this action limit the output of blasts and by so doing induce the suppression of immunity to the antigen by limiting systemic dissemination of specific-antigen

primed blast cells.

The effects of *T. evansi* infection on the changes in output of lymphocyte populations in efferent lymph were similar in many, but also differed in some, respects to what obtains in the peripheral blood. For instance, in the peripheral blood of infected sheep, there is marked decrease in the proportions of all T cell subsets but only a small decrease in their absolute numbers, whilst both the proportions and numbers of B cells showed marked increases. Following primary and secondary vaccination, the proportions of all T cell subsets showed minor variations but their absolute numbers progressively decreased as the cell output decreased. Moreover, although the proportion of B cells increased, their absolute numbers showed little alteration during the first week after vaccination, but thereafter also decreased. In the uninfected sheep, the trend of events in the efferent lymph was similar to the peripheral blood after both primary and secondary vaccinations in that only minor variations occurred in the proportions of the various lymphocyte subsets. However, in absolute numbers there was much larger increase in $CD5^+$, $CD4^+$, $CD8^+$, $\gamma\delta$ T cells as well as B cells in the efferent lymph than in the blood of uninfected sheep following vaccine administration which was a result of the large increases in total cell output.

One of the most surprising findings of this study is the appearance of $CD4^+CD8^+$ cells in the infected sheep, a feature not associated with mature peripheral T cells. However activation-dependent coexpression of CD4 and CD8 antigens has been described for both human and rat polyclonal (Blue *et al.*, 1985; 1986; Green and Jotte, 1985) and clonal (Ottenhoff *et al.*, 1986; Ellerman *et al.*, 1988) T cell populations and for PHA-activated sheep efferent lymphocytes (McClure and Hein, 1989). It is therefore possible that the coexpression of CD4 and CD8 in the infected sheep may have been activation induced. However, two-colour immunofluorescence analysis of Con A-stimulated sheep PBLs (Mackay *et al.*, 1986) and of sheep prescapular efferent lymphocyte after *in vivo* stimulation with either OVA or PPD (Bujdoso *et al.*, 1989) indicates that coexpression of CD4 and CD8 is not a feature of both *in vivo* and *in vitro* activated mature sheep T cells. This is supported by observations in this study, in the uninfected animals, that *in vivo* activation of efferent lymphocytes using *Pasteurella* vaccine did not induce the coexpression of CD4 and CD8 as seen in the infected sheep.

It therefore follows that *T. evansi* possibly provides a secondary stimulus which induces hyperactivation and production of double positive T cells or the increased mobilisation of immature thymocytes. Since extra-thymic T cells showing immature $CD4^+CD8^+$ characteristics are destroyed by activation-induced programmed cell death (PCD) or apoptosis (MacDonald and Lees, 1990; Swat *et al.*,

1991; Young, 1992; Vasquez *et al.*, 1992), it was hypothesised in Chapter 5 that the marked decreases in the proportions of various T cell subsets in the blood of infected/vaccinated sheep may have arisen from cell depletion, by PCD, of cells induced to terminally differentiate into $CD4^+CD8^+$ expression by the trypanosomes.

This hypothesis is supported to some extent by the findings of these experiments. The increase in $CD4^+CD8^+$ cells was later accompanied not only by marked reductions in the absolute numbers of all lymphocyte populations but also, by a reduction in the total and blast cell output from the draining lymph node. Trypanosome infections have been shown to induce cell depletion from the paracortical T cell areas of lymph nodes of infected cattle and sheep (Morrison and Murray, 1979; Mwangi *et al.*, 1991), thus the reduction in cell output may not be a result of prolonged cell shutdown in the node but possibly a combination of cell death and PG action. Moreover, it has also been shown that initial increases in cellular output and in the proportion and numbers of T cell subsets in the efferent lymph of node draining the site of sheep infected with *T. congolense* was followed subsequently by marked decreases in the T cell numbers and proportions (Mwangi, 1991).

These notwithstanding, it could be argued that the increase in double positive cells may be a result of increased mobilisation of immature thymocytes and not a hyperactivation-induced priming of the cells for self-destruction by apoptosis, since there was a marked increase in cells expressing CD1, an antigen normally present on immature thymocytes. If this had been the case, then the coexpression of CD4 and CD8 would also have been greater in the uninfected/vaccinated sheep, since the increase in $CD1^+$ cells was much greater in them. In any case, CD1 is also present on mature B cells and its expression in both groups paralleled those of VPM 30^+ (B) cells. In the absense of a CD4/CD1 and CD8/CD1 two-colour flow cytometry to clarify the issue, one would conclude that the increase in $CD1^+$ cells was a result of B, and not of $CD4^+CD8^+$, cell increase.

Furthermore, since T cell maturation progresses through a phase of $CD4^-CD8^-$ to $CD4^+CD8^+$ state before the final differentiation into mature single positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) cells (Kadish and Basch, 1977; Fowlkes and Mathieson, 1985; Scollay *et al.*, 1988; Guidos *et al.*, 1989) it could be reasoned that the $CD4^+CD8^+$ cells are antigen activated T cells in the process of differentiating into mature single positive cells. An inherent difficulty in this argument is, why if this is so, did the population not increase in the uninfected sheep? Moreover, if they were maturing activated and dividing cells, then the end result would have been that of increases and not of decreases in the cell output and various T cell subsets in the infected animals.

Therefore, it is likely that the appearance of $CD4^+CD8^+$ cells in the efferent lymph of trypanosome-infected sheep, followed by subsequent decrease in various lymphocyte subsets, is an indication that the cells are being depleted by the process of activation-induced cell death. It is not known how trypanosomes bring about the coexpression of these antigens or what finally induces them to self-destruct. However, it is known that when thymocytes are activated in culture, a distinct population coexpressing reduced levels of CD4 and CD8, and which represents cells undergoing PCD arises, and that further activation of the cells by the addition of a second stimulant (ionomycin), accelerates the process of cell death (Swat *et al.*, 1991). Furthermore, the engagement of the TcR complex of immature double positive cells by antigen, initiates the process of PCD (Jenkinson *et al.*, 1989; Smith *et al.*, 1989). It is therefore hypothesised here that whilst trypanosomes by some mechanism(s) trigger the coexpression of CD4 and CD8 by activated T cells, it is the introduction of a second antigen (*Pasteurella* vaccine) which provides the ultimate signal for intrinsic self-destruction of the cells by engaging the TcR complex of the $CD4^+CD8^+$ cells. Trypanosome infection of sheep without a second antigenic challenge, coupled with two-colour immunofluorescence analysis of their efferent lymphocytes should validate or nullify this hypothesis. Similarly, the confirmation as to whether the $CD4^+CD8^+$ cells are actually activated cells driven to spontaneous self destruction by PCD, mobilised immature thymocytes or activated cells undergoing normal differentiation and maturation, will depend on sorting out the cells by FACS, isolating their DNA and analysing it by agar gel electrophoresis. If the cells represent a population dying by apoptosis, then such an analysis should demonstrate the degradation of the DNA into oligonucleosomal fragments characteristic of cells underoing activation-induced death by apoptosis (Jenkinson *et al.*, 1989; Smith *et al.*, 1989; Swat *et al.*, 1991). On the other hand, confirmation as to whether they are immature cells undergoing normal cell division could be carried out by FACS sorting them and looking at the pattern of rearrangement of the T cell receptor genes. If they are normal double positive immature cells they will have far lower proportions of rearrangement patterns of the TcR genes.

A second interesting finding of this study, is the very large increases in the population of $CD5^+$ B cells in the infected animals, which confirms that this cell population is not restricted to the blood (Chapter 5). However, the proportion of this B cell population in the efferent lymph is far more than the blood and in some instances nearly 100 percent of B cells are $CD5^+$. Nevertheless, it has been shown that up to 90 percent of peripheral blood B cells express the CD5 antigen in cattle infected with *T. congolense*, which suggests that increased expression of this cell type could be an important feature of trypanosome infections in animals.

Furthermore, *T. cruzi* infections in mice have been shown to preferentially induce the expansion of $CD5^+$ B cells (Minoprio *et al.*, 1989b).

It is not known how trypanosome infection induces increased expression of this B cell population, especially in the efferent lymph, since it has been shown that $CD5^+$ B cells are not detectable within ovine lymph node or efferent lymph (Mackay, 1988). However, in adult sheep, $CD5^+$ B cells are most numerous in the spleen where up to 20 percent of B cells express the CD5 molecule (Mackay, 1988). It is therefore possible, that following trypanosome infection, there is increased mobilisation and recirculation of $CD5^+$ B cells from the spleen to lymphoid organs where they would not normally be found. On the other hand, it could be that the activation of conventional B cells by the parasites induces them to express the CD5 antigen in large proportions. Support for this comes from observations that *in vitro* activation of conventional human PBL B cells resulted in up to 70 percent of them becoming $CD5^+$ (Miller and Gralow, 1984; Freedman *et al.*, 1987; Werner-Favre *et al.*, 1989). However, the fact that cell transfer experiments in mice have shown that $CD5^+$ B cells have a capacity for self-renewal, suggests that they represent a separate cell lineage distinct from conventional B cells and are not mere activation markers for B cells (Hayakawa *et al.*, 1985; Hardy and Hayakawa, 1986; Herzenberg *et al.*, 1986; Forster and Rajewsky, 1987).

The significance of $CD5^+$ B cell population in the induction of trypanosome-specific immunity or in the immunopathology of the disease is not known and would have to await the characterisation of their antibody production. However, in human and rodent, $CD5^+$ B cells are committed to the production of low affinity, polyreactive IgM auto-antibodies (Hayakawa *et al.*, 1983). Since these heterophile IgM autoantibodies have been demonstrated in trypanosome-infected animals (Henderson-Begg, 1946; Houba *et al.*, 1969; Parratt and Herbert, 1979) it is likely that the $CD5^+$ B cells may be responsible for their production. If this is so, then it would add to the explanation not only of the reported increase in immunoglobulin destruction occurring in infected animals (Nielsen *et al.*, 1978a, 1978b) but also of the reduction in cell numbers as already shown. This is because the polyreactive IgM autoantibodies produced by $CD5^+$ B cells have been shown to bind both to Fc fragment of IgG antibodies and to thymocytes (Hayakawa *et al.*, 1984; Casali *et al.*, 1987; Hardy *et al.*, 1987; Burastero *et al.*, 1988; Nakamura *et al.*, 1988). It would therefore follow that since the primary target *in vivo*, of anti-TcR autoantibodies is the $CD4^+CD8^+$ cells (Smith *et al.*, 1989) the increased production of polyreactive autoantibodies by the increasing numbers of $CD5^+$ B cells following trypanosome infection would result in increased destruction of the equally increasing $CD4^+CD8^+$ cell population. Similarly, binding of the Fc fragments of the

IgG antibodies would result in the increased Ig catabolism reported.

Given the persistence of this cell population in infected animals in addition to the alterations in T cell subsets the net result of these sequence of events would be generalised immunosuppression as demonstrated in infected animals. Obviously a lot more work is needed to elucidate fully the exact implications of the observations made in this study in the immunopathology of trypanosome infection. For instance, the assay of the type and level of cytokine production in the lymph of infected animals would have given a fairly clear indication as to which T cell subset is being suppressed or activated by the infection and would have helped in explaining the eventual failure in both T and B cell functions. It would have also been useful to assay for *Pasteurella* and parasite-specific antibodies in the lymph of the animals. Unfortunately, neither the assay of T cell derived lymphokines nor of antigen-specific antibody production in the efferent lymph was possible due to the loss of the entire batch in an arson attack on CTVM. Such assays would have provided further evidence as to whether the phenotypic changes observed affected the parasite or *Pasteurella* specific antibody responses in the lymph as was observed in the serum.

CHAPTER SEVEN

Trypanosome-induced Immunosuppression: The Role of Macrophages and Suppressor T Cells

7.1 Introduction

One of the hallmarks of African trypanosomosis in rodents is the induction of profound immunosuppression to a variety of antigens, with both B and T cell functions impaired. *In vitro*, the impaired B and T cell functions is shown by profound reduction in DNA synthetic responses of leucocytes to stimulation with such mitogens as pokeweed mitogen (PWM), Con A and LPS (Goodwin *et al.*, 1972; Corsini *et al.*, 1977; Jayawardena and Waksman, 1977; Pearson *et al.*, 1978; Roelants *et al.*, 1979a, b; Gasbarre *et al.*, 1981). Moreover, similar lympho-proliferative experimental assays, employing mononuclear cells derived from rodent spleen and lymph node organs, have been used to extensively study the underlying mechanisms surrounding the generalised immunosuppression. Evidence from these studies suggests that the suppression is multifactorial and may include: exhaustion of antigen-reactive B cells which are driven to terminal differentiation (Greenwood, 1974; Hudson *et al.*, 1976; Corsini *et al.*, 1977), generation of suppressor T cells (Eardley and Jayawardena, 1977; Jayawardena and Waksman, 1977; Jayawardena *et al.*, 1978) and generation of suppressor macrophages (Corsini *et al.*, 1977; Wellhausen and Mansfield, 1979; Sileghem *et al.*, 1989a, b; Borowy *et al.*, 1990). Suppressor T cells and macrophages produce cytokines and prostaglandins respectively, which act in a series of complex immunoregulatory mechanisms to suppress the capacity of helper T cells to provide help for antigen-specific responses by B cells (Alcina and Fresno, 1985; Sileghem *et al.*, 1989a, b; Darji *et al.*, 1991a, b; Nabors and Tarleton, 1991; Sileghem *et al.*, 1991).

In the case of cattle, infection with pathogenic trypanosomes has also been shown to cause suppression of systemic humoral responses to bacterial and viral vaccine antigens (Scott *et al.*, 1977; Whitelaw *et al.*, 1979; Rurangirwa *et al.*, 1983). However, attempts to investigate the underlying mechanisms using *in vitro* cell culture systems are limited and have not gone beyond the assessment of the responsiveness of mononuclear cells from infected animals to mitogenic or homologous trypanosomal antigenic stimuli *in vitro*. Results from such studies have demonstrated suppression of the responsiveness of these cells to stimulation with either Con A, PWM, LPS or homologous trypanosomal antigen (Emery *et al.*, 1980c; Masake *et al.*, 1981). This suppression is associated with active infection only and is abrogated by treatment of the infected animals with a trypanocidal drug. This suggests a role for trypanosome antigens in the immunosuppression. However, cells from cattle previously immunised with formalin-fixed *T. congolense* responded to subsequent specific stimulation with the ultrasonicated homologous antigen (Emery *et al.*, 1980), suggesting that other mechanisms apart from the presence of trypanosomal antigens *per se* may be operative. Moreover, it has recently been

shown that cells from *T. congolense*-infected Boran and N'dama breeds of cattle can actually proliferate in response to stimulation with soluble homologous *T. congolense* antigen even though the response is transient (Flynn, Sileghem and Williams, 1992). Hence, the mechanisms underlying the immunosuppression seen in trypanosome infected ruminants still remain under-investigated and poorly understood.

Recent studies have shown that infection of cattle with *T. congolense* causes alterations in their cell population dynamics which possibly underlies the immune dysfunction seen (Ellis *et al.*, 1987; Mwangi, 1991; Williams *et al.*, 1991). Moreover, infection of sheep with *T. evansi* precipitates substantial reductions in the proportions of effector T cell subsets which is accompanied by persistent expansion of circulating B cells and suppression of humoral responses to primary and secondary inoculations with *P. haemolytica* vaccine antigen (Chapters 4 and 5). Although the reductions in the proportions of these cell phenotypes in the blood are marked, their absolute numbers show relatively smaller reductions and yet suppression of antibody responses to the vaccine antigen occurs. In sheep which had undergone selfcure, where the changes in both the proportions and numbers of the various lymphocyte subsets are minimal, suppression still occurs. Furthermore, even after selfcure when trypanosomes have been eliminated, secondary responses to *Pasteurella* vaccine are not restored to the levels seen in uninfected, control sheep. These findings suggest that in addition to the role of parasite antigens and alterations in the dynamics of various lymphocyte surface molecules, other mechanism(s) might operate in compromising the immune competence of infected livestock.

This chapter describes *in vitro* lymphocyte transformation assays using T and B cell mitogens and specific antigenic stimuli to provide further information on factors contributing to the immunosuppressive effects of *T. evansi* in infected sheep. In addition, since suppressor T cells and macrophages have been shown to play central roles in the immunosuppression seen during rodent trypanosomosis (Askonas, 1985; Sileghem *et al.*, 1989a; b; 1991; Bakhiet *et al.*, 1990; Borowy *et al.*, 1990), and in cattle infected with *T. congolense* (Flynn and Sileghem, 1991), experiments were also carried out to deplete these cell types from the cell population prior to initiation of cultures. This was in order to assess whether they play any role in *T. evansi*-induced immunosuppression in sheep.

7.2 Materials and Methods

7.2.1 Animals, Vaccination, Infection and Experimental Design

Six Suffolk sheep, housed and fed as described in Section 3.1 were used in these studies. Four animals, 225, 476, 443 and 766, were each primed and boosted with 2 mg dose of *Pasteurella* vaccine four weeks apart as described in Section 3.8.1. Two weeks after secondary vaccination, sheep 443 and 766 were each infected intravenously with 2×10^6 *T. evansi* TREU 2143 using the external jugular vein. Sheep 225 and 476 were used as uninfected, vaccinated controls. The mitogenic and antigen specific responses of peripheral blood leucocytes from these animals were tested in *in vitro* cell culture assays. Assays were initiated 7 days after infection in both infected and control sheep and subsequently were conducted weekly until three weeks p.i., when the infected animals were treated with Cymerlarsan. Thereafter, three further assays were conducted in the infected/treated sheep at ten day intervals in order to assess the effect of trypanocidal drug therapy on the cell responses.

In a follow-up experiment, two more sheep, 222 and 396 were vaccinated and infected as described above. Cells from these sheep were used in depletion assays in order to assess the effects of removal of $CD8^+$ and monocyte cell populations on proliferative responsiveness to mitogenic and antigenic stimulation *in vitro*. $CD8^+$ cells and monocytes were depleted as described in Sections 3.9.2.2 and 3.9.2.3 respectively. Assays were initiated three weeks prior to infection and thereafter were conducted every four days for 40 days p.i. and subsequently at ten day intervals until 80 days after infection. Treatment with Cymelarsan was on day 50 p.i.

7.2.2 Mitogens, Antigens and Cell Cultures

Con A and LPS were used as T and B cell mitogens respectively. *P. haemolytica* A1 capsular antigen, live *T. evansi* TREU 2143 or soluble trypanosomal antigens, prepared as described under Sections 3.8.2 and 3.10.1, were used as specific antigenic stimuli. Both the mitogens and the antigens were freshly reconstituted to their various working dilutions in RPMI-1640 complete culture medium (Appendix II[7]) shortly before use. The cell culture systems were optimised as described in Section 3.9.3.

Ovine peripheral blood leucocytes were prepared from defibrinated venous blood by density gradient centrifugation over Lymphoprep as described in Section 3.10.2 and Appendix II(2), and adjusted to 10^7 cells ml^{-1} using complete culture medium. For the assays, triplicate cultures were set up in 96-well flat-bottomed NUNC microculture plates in a final volume of 200 µl RPMI-1640 complete

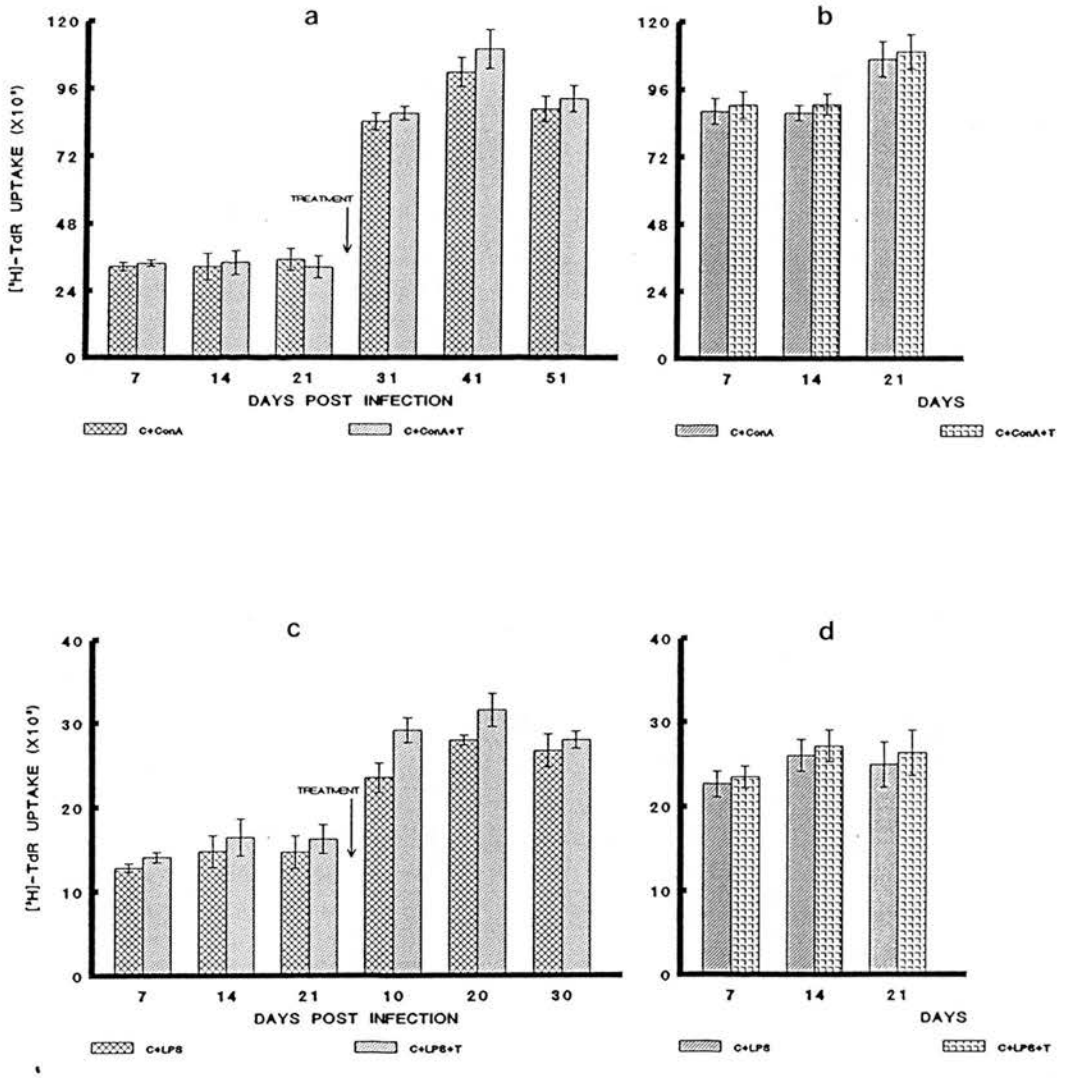


FIGURE 7.1 Proliferative responses of PBLs of *T. evansi* infected and uninfected sheep to *in vitro* stimulation with Con A and LPS in the presence or absence of live or soluble homologous *T. evansi* antigen.

(a) Responses to Con A in infected sheep
(b) Responses to Con A in uninfected sheep
(c) Responses to LPS in infected sheep
(d) Responses to LPS in uninfected sheep

In each case values represent mean ± S.D. of triplicate cultures for each treatment from 2 infected and two uninfected sheep. In cocultures of cells with either Con A or LPS and *T. evansi* antigen, soluble parasite antigen was used on days 14 p.i. (for both infected and uninfected sheep) and on day 20 after treatment (infected sheep only) while live organisms was used at all other times.

culture medium as described under Section 3.9.4. Cells were dispensed in 5 µl volumes to give a final cell concentration of 10^5 cells per well (5×10^5 cells ml^{-1}) and stimulated with either 2.5 µg ml^{-1} Con A, 75 µg ml^{-1} LPS, 50 µg ml^{-1} P. ag and/or 5×10^5 *T. evansi* TREU 2143 or 12.5 µg ml^{-1} of its soluble antigen (T. ag). Unstimulated cell cultures were also set up, as well as cultures of live trypanosomes with Con A and LPS to assess the possible uptake of these mitogens by the trypanosomes. Cultures were incubated, pulsed with thymidine, harvested and counted as described in Section 3.9.3.1 and results presented as mean counts per minute (CPM) of the cultured cells and trypanosomes.

7.3 Results

7.3.1 Cell Responses to Con A and LPS Stimulation

The proliferative responses to both mitogens of peripheral blood leucocytes from *T. evansi*-infected sheep were substantially reduced when compared with uninfected controls. This reduction was associated with active infection as the responses were restored after trypanocidal drug treatment of infected sheep (Figure 7.1a, c). To assess whether live trypanosomes or soluble trypanosomal antigen exert similar depression *in vitro*, 5×10^5 parasites or 12.5 µg ml^{-1} of soluble antigen were added to the Con A or LPS stimulated cells from infected and uninfected control sheep. Results showed that at these concentrations, *T. evansi* or its soluble antigen neither suppresses the responsiveness of cells from uninfected sheep to Con A or LPS stimulation nor exacerbates the depression seen in cells from actively infected sheep. On the contrary, a small relatively insignificant mitogenic effect was observed as slightly higher thymidine uptake was observed in cells cocultured with live or soluble *T. evansi* antigen than in cells cultured with Con A or LPS alone (Figure 7.1a, b, c & d). However, a separate experiment showed that at higher concentrations, live but not soluble *T. evansi* antigen depressed the ability of cells from normal uninfected sheep to proliferate *in vitro* when stimulated with Con A or LPS (Table 7.1). Furthermore, to assess whether the depression was as a result of live trypanosomes depleting Con A or LPS during culture, 8×10^6 parasites ml^{-1} were cocultured in a FALCON tissue-culture flask with 2.5 µg ml^{-1} Con A or 75 µg ml^{-1} LPS in a total volume of 8 mls RPMI-1640 complete culture medium. At the end of 72 hour culture, the parasites and debris were removed by centrifugation and the supernatants used to stimulate cell cultures from normal sheep without further addition of mitogens. Table 7.2 shows that enough Con A and LPS concentrations were left in the supernatants to induce marked proliferative responses in the cells. Moreover, cultures of parasites alone pulsed with thymidine and counted in a liquid scintillation counter, showed that *T. evansi* did not incorporate thymidine (Table

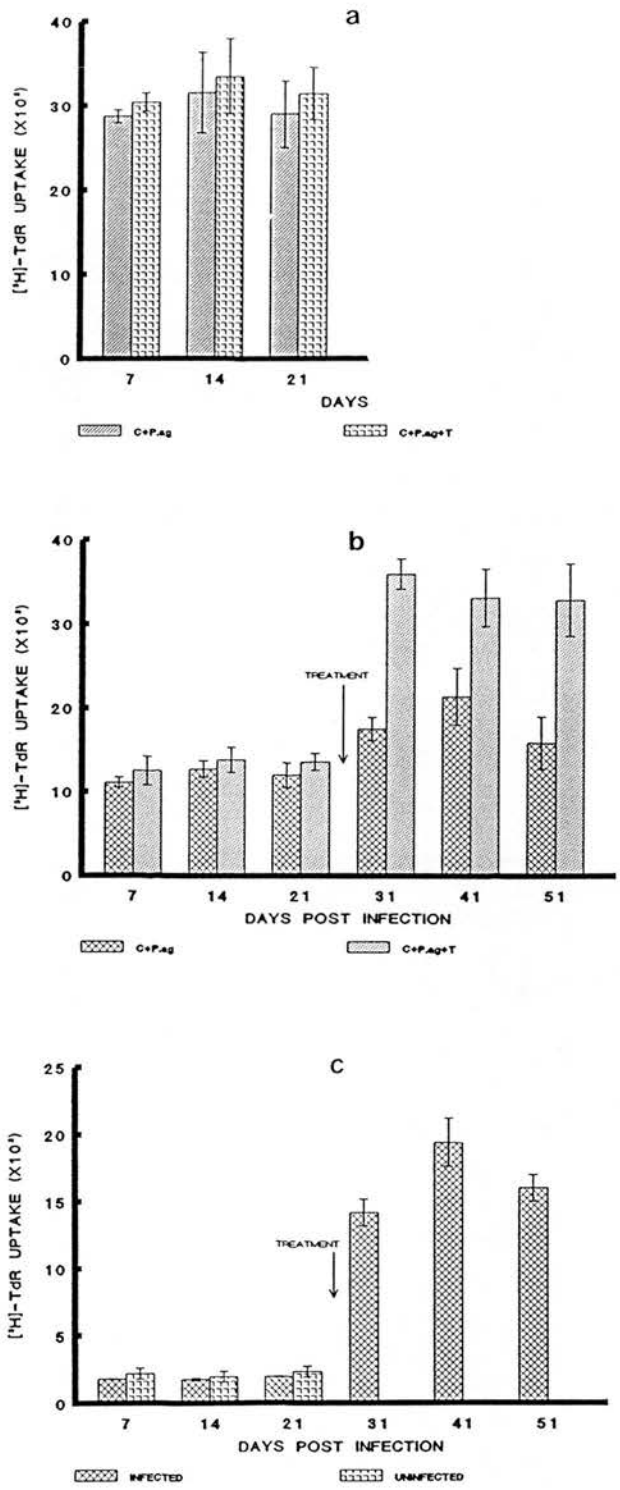


FIGURE 7.2 Proliferative responses of PBLs of *Pasteurella* vaccine immunised/*T. evansi* infected and uninfected sheep to specific *in vitro* stimulation with *Pasteurella* and *T. evansi* antigens. (a) Response to P.ag in uninfected sheep, (b) Response to P.ag in infected sheep, (c) Responses to stimulation with live or soluble *T. evansi* antigens in infected and uninfected sheep. In all cocultures with trypanosomal antigen, soluble antigen was used on days 14 p.i. and 20 post treatment while live organisms was used at all other times.

7.2).

7.3.2 Cell Responses to *Pasteurella* Antigen

The effect of *T. evansi* infection on the ability of PBLs from sheep previously immunised with *Pasteurella* vaccine to proliferate when stimulated *in vitro* with the specific P. ag was tested. Cells from infected animals were essentially unresponsive to stimulation with P. ag alone or to stimulation with a combination of P. ag and live or soluble T. ag *in vitro* (Figure 7.2b) when compared with that of the vaccinated control sheep (Figure 7.2a). This defect was abrogated by anti *T. evansi* treatment, although the responses observed were still less than those of the control sheep. However, treatment resulted in an additive effect on the responsiveness of cells co-stimulated with P. ag and live or soluble T. ag (Figure 7.2b).

7.3.3 Cell Responses to Trypanosomal Antigens

The responsiveness of PBLs from infected and uninfected sheep to stimulation *in vitro* with homologous trypanosome or the soluble antigen was also tested. Cells from infected sheep failed to respond to stimulation with either antigen. This suppressive effect was abrogated by trypanocidal drug treatment. Cells taken 10, 20 and 30 days after treatment showed significant increase in responsiveness to these antigens (Figure 7.2c). At no time did cells from the uninfected control sheep respond significantly to stimulation with either of the antigens (Figure 7.2c).

7.3.4 The Role of $CD8^+$ Cells and Monocytes in *T. evansi*-induced immunosuppression

In an attempt to elucidate further the mechanisms responsible for the *T. evansi*-induced immunosuppression, monocytes and/or $CD8^+$ (suppressor) T cells were deleted from the cells obtained from infected sheep prior to culture with mitogens or antigens. *T. evansi* infection resulted in the depression of responses to Con A, LPS and P. ag stimulation.

The depletion of $CD8^+$ cells resulted in a transient restoration of the responsiveness of cells to Con A stimulation between day 12-20 p.i. (Figure 7.3a, b). At other times, the responses of cell populations depleted of this cell type were similar to those of the undepleted cell population (Figure 7.3a, b). The removal of an adherent cell population (monocytes) produced a greater proliferative response to Con A stimulation between days 4-32 p.i. Between days 36-50 p.i. and after drug treatment, there was no difference between the responses of cells depleted of

Table 7.1 **Effect of increasing numbers and concentration of live or soluble *T. evansi* antigens respectively on the proliferative responses of peripheral blood leucocytes from normal sheep to Con A and LPS *in vitro*. 5 x 10^5 cells ml^{-1} were cultured in the presence of different concentrations of live or soluble *T. evansi* antigen and stimulated with either Con A or LPS.**

Con A responses			
Trypanosome Antigen Concentrations		Mean C.P.M. ± S.D.	
Live (no. ml^{-1})	Soluble (µg ml^{-1})	Live	Soluble
2.5 x 10^5	12.5	77,024.8±3839.8	84,066.2±4499.9
5 x 10^5	25.0	81,363.0±4662.2	83,496.0±4081.9
10^6	50.0	60,329.0±4605.0	83,009.2±4047.4
2 x 10^6	100.0	24,709.0±2742.7	83,523.8±6801.9
4 x 10^6	200.0	19,943.1±2435.9	77,621.6±5339.6
8 x 10^6	400.0	15,873.9±1159.0	69,839.8±3818.5

LPS responses			
Trypanosome Antigen Concentrations		Mean C.P.M. ± S.D.	
Live (no. ml^{-1})	Soluble (µg ml^{-1})	Live	Soluble
2.5 x 10^5	12.5	52,929.3±3741.8	52,536.3±3980.6
5 x 10^5	25.0	57,356.3±4203.6	51,439.9±3949.6
10^6	50.0	49,543.9±2714.0	51,323.7±2872.2
2 x 10^6	100.0	11,798.8±1491.0	46,058.9±2705.6
4 x 10^6	200.0	4,850.2± 440.8	43,651.3±1542.3
8 x 10^6	400.0	4,148.3± 268.0	43,651.3± 990.7

Table 7.2 **Uptake of mitogens by *T. evansi* and the effect on cell responses. 8 x 10^6 *T. evansi* TREU 2143 were cultured for 72 hours in 8 mls of RPMI-1640 complete culture medium containing either 2.5 μg ml^{-1} Con A or 75 μg ml^{-1} LPS. Aliquots of 5 x 10^5 trypanosomes were then pulsed with 3H-thymidine and DNA synthesis determined in a liquid scintillation counter. Trypanosomes were removed from the rest by centrifugation and the supernatant used to culture 5 x 10^5 PBLs from normal sheep, and DNA synthesis determined as above. Three experiments were conducted and results are presented as mean cell count per minute (CPM) ± S.D. of five culture replicates per experiment.**

Cell/Parasite concentration	Experiments	Mean C.P.M. + S.D. Con A	LPS
68.4	1	78,271.3±3,072.0	47,478.1± 8
5 x 10^5 cells ml^{-1} 39.9	2	74,321.7±3,009.1	34,265.5±25
37.2	3	78,224.2±6,465.1	42,529.6±32
5 x 10^5 6.2	1	129.5 ± 11.4	63.8 ±
T. evansi ml^{-1} 6.4	2	134.9 ± 5.6	60.9 ±
49.1	3	139.3 ± 12.9	105.1 ±

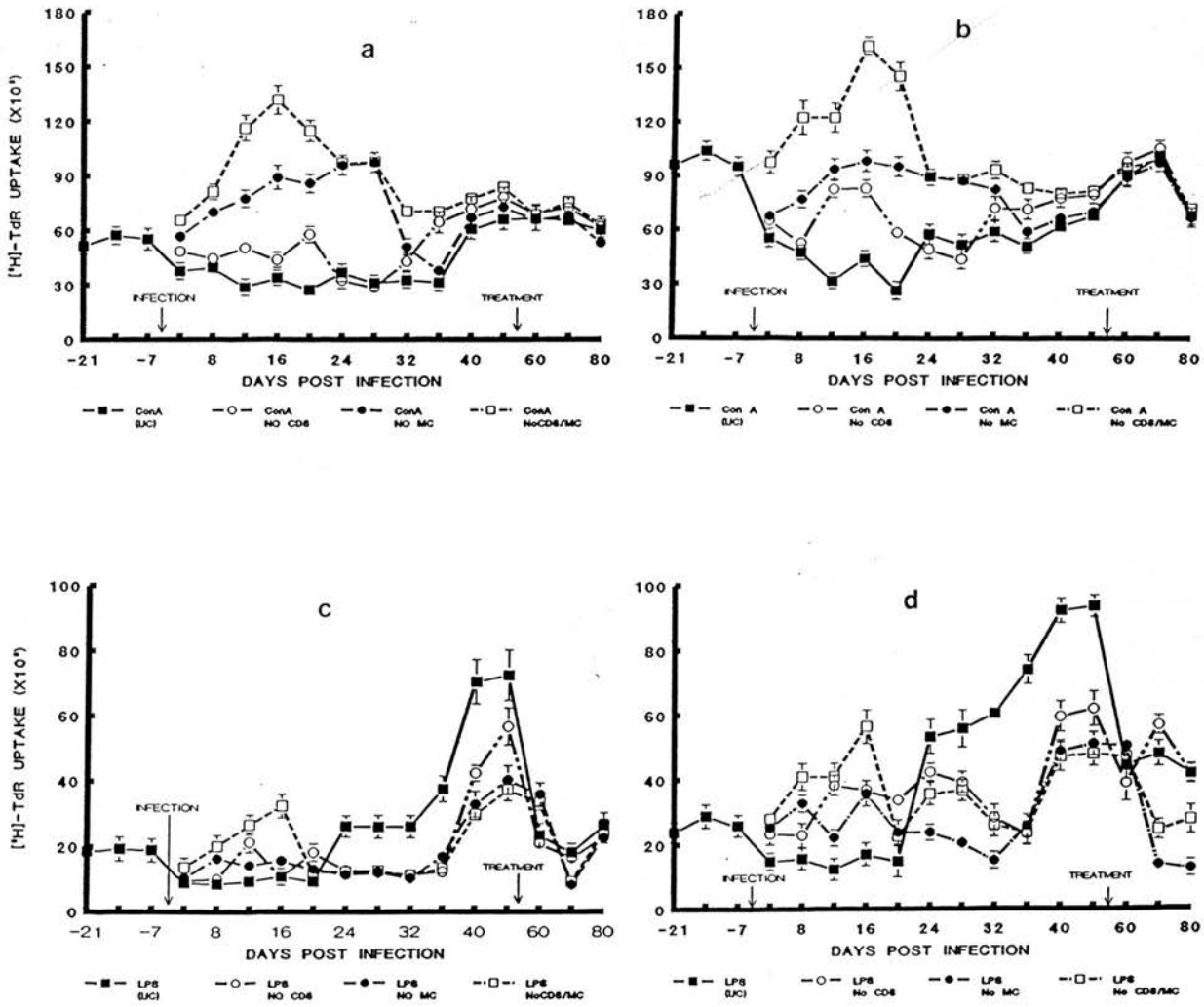


FIGURE 7.3 Effect of the depletion of $CD8^+$ T cells and/or monocytes on the proliferative responses of PBLs of *T. evansi* infected sheep to *in vitro* stimulation with Con A and LPS. (Values are Mean ± S.D. of triplicate cultures in each animals).

(a) Effect of CD8 and/or monocyte removal on the responses of cells from sheep 222 to Con A.

(b) Effect of CD8 and/or monocyte removal on the responses of cells from sheep 396 to Con A.

(c) Effects on responses to LPS in sheep 222.

(d) Effects on responses to LPS on sheep 396.

MC = Monocytes
UC = Undepleted Cells

monocytes and undepleted cells. Simultaneous removal of both $CD8^+$ cells and monocytes produced a greater enhancement in the responsiveness of the cells to Con A between days 4-20 p.i. Between days 24-32 p.i., the responses of cells depleted of both cell types were similar to those depleted of monocytes only. By day 40-50 p.i., the responses to Con A of all cell populations, whether depleted or not, were similar (Figure 7.3a, b).

The depletion of either $CD8^+$ cells or monocytes or removal of both cell types simultaneously, restored the responsiveness to LPS stimulation of PBLs from sheep 396 (Figure 7.3d). However, the effect in sheep 222 was transient and limited (Figure 7.3c). From day 25 p.i. onwards until after drug treatment, the responsiveness of the undepleted cell population to LPS stimulation increased by up to 4-fold exceeding, by a considerable margin, the responses obtained with the depleted cell populations during the same period (Figure 7.3c, d). Following treatment, the responses returned to levels similar to those obtained prior to infection.

Depletion of the $CD8^+$ T cells resulted only in a transient restoration of cell responses to P. ag stimulation between days 12-20 p.i. Thereafter, $CD8^+$ cell depletion had no effect and in fact, responses obtained with the undepleted cell population slightly exceeded those of the depleted population (Figure 7.4a, b). Both responses however, remained below pre-infection levels until after treatment when an enhanced proliferative response to P. ag stimulation was obtained.

Parasite-specific T cell responses remained suppressed throughout the period of infection except for day 12 and 16 p.i. (sheep 222, Figure 7.4c) and day 8-16, 24-28 p.i. (sheep 396, Figure 7.4d) when a slight increase was observed in cells depleted of $CD8^+$ T cells. Following treatment, more than 8-fold increase was observed in the proliferative responses of PBLs to stimulation with homologous trypanosomal antigen whether the $CD8^+$ cells were depleted or not (Figure 7.4c, d).

7.4 Discussion

In rodent trypanosomosis, it is generally agreed that 'suppressor' cells arise during the infection. Several reports employing *in vitro* co-culture and cell transfer experiments showed that these cells are either T cells or macrophages and that the immunosuppression seen during infection is mediated either by these suppressor macrophages, T cells or both (Roelants and Pinder, 1984; Askonas, 1985; Bancroft and Askonas, 1985; Sileghem *et al.*, 1986). The contradictory nature of these reports prevented the unequivocal identification of the cell type to which the suppressor cells belong. Recent studies, employing more sophisticated and efficient cell separation or depletion methods, have confirmed that although $CD8^+$ T cells do

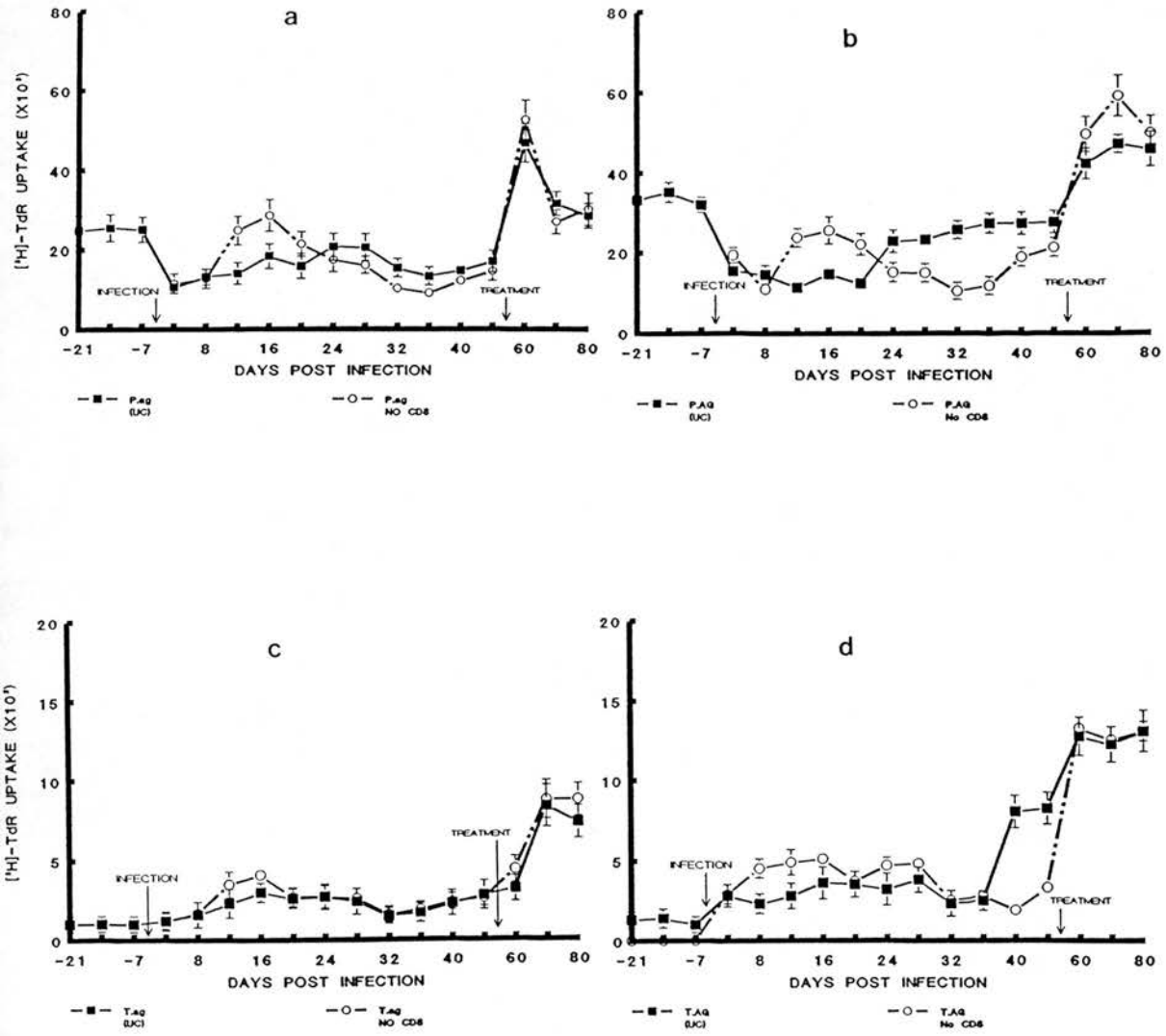


FIGURE 7.4 Effect of the depletion of $CD8^+$ cell population on the proliferative responses of PBLs of *T. evansi* infected sheep to *in vitro* stimulation with *Pasteurella* and trypanosomal antigens.

(a)	Effect on cells from sheep	222 to P.ag
(b)	" " " " "	396 to P.ag
(c)	" " " " "	222 to T.ag
(d)	" " " " "	396 to T.ag

Values are Mean ± S.D. of triplicate cultures.

play some role (Bakhiet *et al.*, 1990), the cells which play the central role in the induction of generalised immunosuppression during rodent trypanosomosis are macrophages (Sileghem *et al.*, 1989b; Borowy *et al.*, 1990; Darji *et al.*, 1991a, b; Sileghem *et al.*, 1991; Cerrone *et al.*, 1992). In addition, it has also been shown that the depletion of the monocyte-macrophage population of cells derived from the lymph node or peripheral blood of *T. congolense*-infected cattle, effectively abrogated the previously observed immunosuppression to Con A stimulation (Flynn and Sileghem, 1991). This indicates that macrophages/monocytes also play a key role in the induction of trypanosome-associated immunosuppression in cattle.

Results presented in this chapter show that *T. evansi* infection in sheep depresses the responsiveness of PBLs to mitogenic and antigenic stimuli *in vitro*. The decrease in Con A and LPS responses are similar to those described by Masake *et al.* (1981) in which the stimulatory effects of LPS, PWM and Con A on PBLs from *T. congolense*-infected cattle were significantly decreased. Co-culture of 5×10^5 trypanosomes ml^{-1} with PBLs from normal sheep did not depress the responses of the cells to mitogens or specific antigen. This also confirms the work of Masake *et al.* (1981) in which co-cultivation of 2×10^5 live *T. congolense* with 2×10^6 PBLs ml^{-1} from normal cattle, did not depress the responsiveness of the cells to Con A stimulation. However, subsequent results obtained in the present study showed that higher concentrations of parasites were able to induce the suppression of proliferative responses to mitogenic stimulation. Such suppression was not as a result of the exhaustion of either Con A or LPS by the large population of trypanosomes as trypanosomes failed to incorporate any of the mitogens to any measurable extent. Moreover, culture medium supernatant obtained after culture of 8×10^6 trypanosomes ml^{-1} for 72 hours, and which originally contained optimal concentrations of Con A and LPS, was still able to induce cell proliferation to levels comparable to those obtained with cells cultured in freshly prepared medium containing optimal concentrations of mitogen. This result supports observations in rodents which show that the degree of immunosuppression is dependent upon the level of parasitaemia, being more severe in hosts that have high parasitaemias and least marked in hosts that have low parasitaemias (Vickerman and Barry, 1982). In addition, this supports earlier results in this thesis (Chapters 4 and 5) in which it was observed that although *T. evansi* infections in sheep are generally chronic with low or cryptic parasitaemias, the suppression of parasite-specific and anti-*Pasteurella* antibody responses was greater in sheep in which parasitaemia was relatively high enough to be detectable by HCT throughout the course of the infection.

Suppression of responses to stimulation with either Con A, LPS or

Pasteurella antigen is associated with active infection with *T. evansi* and cell proliferative responsiveness is restored after treatment with trypanocidal drug. Moreover, cells from infected animals failed to respond to stimulation with soluble homologous trypanosomal antigen until after treatment. These results are in agreement with those obtained using *T. evansi*-infected ponies in which PBLs failed to respond to different concentrations of a soluble fraction of the parasite until the animals were treated (Ahmed *et al.*, 1988). Furthermore, the results are similar to what was obtained with *T. congolense* infected cattle, in which PBLs failed to exhibit specific blastogenesis *in vitro* in response to incubation with ultrasonicated *T. congolense* antigen, while cells from infected/treated cattle responded normally (Emery, *et al.*, 1980). However, those and the present results contrast with a recent report claiming that mononuclear cells from the blood and spleen of *T. congolense*-infected Boran and N'dama breeds of cattle exhibited antigen-specific proliferation when stimulated *in vitro* with a soluble protein lysate of the homologous parasite (Flynn, *et al.*, 1992). It is noteworthy that the antigen-specific responses observed by these authors was transient and also very inconsistent, occurring either on day 21 or 28 on one occasion and on day 7, 14 or 20 on another, after which the responses either disappeared or remained detectable at very low levels. Moreover, lymph node cells from these animals did not show any responsiveness to specific trypanosomal antigen.

Although in this study, the blastogenic responses of cells from vaccinated/infected sheep to stimulation with Con A, T. ag and to some extent P. ag remained depressed throughout the period of infection, the responsiveness of the cells to LPS was not only restored from day 24 p.i., but was much higher than the preinfection levels. In addition, while responses to Con A, T. ag and P. ag rose after treatment, that of LPS decreased. This is probably explained by the fact that LPS is a B cell-specific mitogen and that in *T. evansi* infected sheep there is marked B cell lymphocytosis (Chapters 4 and 5). Similarly, there is lymphoid organ hyperplasia and increased proliferation of B cells in trypanosome-infected cattle and sheep (Morrison and Murray, 1979; Masake and Morrison, 1981; Ellis *et al.*, 1987; Mwangi *et al.*, 1990, 1991; Mwangi, 1991; Williams *et al.*, 1991). It is therefore possible that the increased response to LPS stimulation is a reflection of the non-specific B cell activation and expansion resulting from failure in regulatory T cell function. Interestingly, this responsiveness to LPS stimulation commenced at about the same period of infection when increased B cell responses were observed in the blood of *T. evansi*-infected sheep (Chapters 4 and 5). On the other hand, the depression in Con A and specific antigen T cell responses could be due to the decrease in the proportion of T cells in infected sheep since *T. evansi* infection results in marked

decreases of T cell subsets (Chapters 4 and 5).

Cell depletion experiments showed that although $CD8^+$ T cells and cells of the monocyte/macrophage lineage both play a role in the *T. evansi*-induced suppression of blastogenic responses to mitogens and antigens, the latter cell type seems to be the main mediator of suppression. Restoration of responsiveness to Con A and LPS stimulation was less in cells depleted only of $CD8^+$ cells and was more limited and transient in the case of antigen-specific responses. The depletion of $CD8^+$ T cells *in vivo* in rats infected with *T. brucei* by intraperitoneal injection of anti-CD8 mAb was shown to have suppressed parasite growth and prolonged survival of infected rats but failed to induce selfcure and prevent death of the rats (Bakhiet *et al.*, 1990). This effect was said to have resulted from the abrogation of IFN-γ production in the CD8-depleted rats, and it was suggested that $CD8^+$ T cells are directly or indirectly involved in the induction of the immunosuppression seen during the infection (Bakhiet *et al.*, 1990). However, since the rats failed to control the parasitaemia, with all of them dying subsequently, it suggests that although $CD8^+$ cells may be involved in the immune dysfunction, their role may be limited and secondary to other suppressive factors. Moreover, with the human parasite *T. cruzi*, spleen cells collected from acutely or chronically infected mice and depleted of Lyt 2.1^+ cells by mAb treatment and complement lysis, failed to produce a better response than that mounted by untreated cells when stimulated with lysed homologous *T. cruzi* antigen (Kierszenbaum, 1981). This report is similar to the finding in the present study since the removal of $CD8^+$ cells had little or no effect on improving the responsiveness of treated cells to *Pasteurella* or trypanosomal antigen stimulation.

The effect of the removal of monocytes on the responsiveness of the cells to Con A and LPS stimulation clearly shows that monocytes are the predominant suppressive cell types during the infection. Depletion of monocyte/macrophage population from the mononuclear cells of *T. congolense*-infected cattle, has also been shown to restore their proliferative response to Con A stimulation (Flynn and Sileghem, 1991). In rodent models, it has been established with a reasonable degree of confidence, that macrophages have a central role in the progressive immunosuppression induced by trypanosome infections (Grosskinsky and Askonas, 1981; Wellhausen and Mansfield, 1980; Askonas, 1985; Bancroft and Askonas, 1985; Sileghem *et al.*, 1989b; 1991; Borowy *et al.*, 1990; Darji *et al.*, 1991a, b; Cerrone *et al.*, 1992). The findings of the present study are consistent with these reports, indicating that macrophages are important in *T. evansi* immunosuppression in sheep. Although these results demonstrate the importance of macrophages, they do not in any way preclude other immunosuppressive mechanisms, as the complex

aberrations in immune functions observed during trypanosomosis cannot be fully explained by, or assigned to, a single empirical event.

The question of how macrophages mediate suppression still remains. Some of the hypotheses included impaired processing and faulty presentation of trypanosome antigens, and altered secretion of macrophage-derived immunoregulatory factors. It has been shown however, that macrophage function is not lost during the infection and that particulate antigens, including trypanosome organisms, can be internalised and degraded by macrophages during infection (Murray *et al.*, 1974b; McAskill *et al.*, 1981; Askonas, 1985). However, prostaglandins (PGE_1 & E_2), superoxide anion and hydrogen peroxide secretion by macrophages are increased at the peak of parasitaemia (Grosskinsky *et al.*, 1983; Fierer, Salmon and Askonas, 1984; Askonas, 1985), and the secretion of interleukin 1 (IL-1) from macrophages derived from infected mice is also markedly increased (Sileghem *et al.*, 1989c). Prostaglandins of the E series (PGE_1 and PGE_2) have been widely implicated as potent suppressors of both B and T lymphocyte activation and proliferation (Smith *et al.*, 1971; Kurland *et al.*, 1977; Goodwin *et al.*, 1977; Kingston and Ivanyi, 1979; Hopkins *et al.*, 1981b). Moreover, the action of PGs is not limited to the suppression of cellular activation and mitogenesis but has been shown to inhibit a variety of lymphocyte effector functions, including lymphokine secretion (Gordon *et al.*, 1976) and direct cytolysis (Henney *et al.*, 1972; Melmon *et al.*, 1974). In addition, PGs are not only suppressive to lymphocyte effector functions but also inhibit unstimulated and stimulated lymphocyte random migration (McCarty and Goetzl, 1979; Hopkins *et al.*, 1981a; Van Epps, 1981).

Based on these findings it has been proposed that macrophages mediate immunosuppression in trypanosome infected rodents by at least two separate effector mechanisms which block different T cell regulatory steps (Sileghem *et al.*, 1986; 1989a, b; Tarleton, 1988b). Firstly, the PG produced by the trypanosome-pulsed macrophages directly inhibits IL-2 secretion by $CD4^+$ T cells which in turn results in the suppression of T cell proliferation. Secondly, through a PG-independent mechanism, the pulsed macrophages trigger lymphocytes to a faster production of IFN-γ which inhibits IL-2R expression on $CD4^+$ and $CD8^+$ T cells (Sileghem *et al.*, 1987b; 1989a, b; 1991; Tarleton, 1988b; Darji *et al.*, 1991a, b). The increased amount of IL-1 produced by macrophages activates further synthesis of PG (Pentreath, 1991) and IFN-γ synergises this action through its macrophage activating property by stimulating further PG release and thus aggravating the immunosuppression seen (Darji *et al.*, 1991a, b). Whether this sequence of events takes place in *T. evansi*-infected sheep or *T. congolense*-infected cattle is too early to speculate. Certainly more work is required on the effects of different cell types on

the immunosuppression seen.

Finally, results in this chapter have shown that infection of sheep with *T. evansi* depresses the responsiveness of their PBLs to *in vitro* mitogenic and antigenic stimuli, and that the depression is associated with active infection. Treatment of infected sheep abrogates the suppressive effective. Moreover, examination of the roles of $CD8^+$ (suppressor) T cells and monocytes by cell depletion assays showed that the latter cell type is the predominant mediator of suppression.

CHAPTER EIGHT

Concluding Remarks

Despite the emphasis on the study of trypanosome immunopathology, the principles underlying the induction of the generalised immunopathological consequences in trypanosome infected livestock remains poorly understood. One of the main reasons for this is that the majority of current efforts in this area have concentrated on "immunity" to the parasite during the course of infection, and have largely ignored the "immunology" of the infectious process. That is the general alterations in the host immune effector cells orchestrated by the infection. Furthermore, although a considerable amount of literature dealing with the immunity of infections caused by pathogenic trypanosomes now exists, relatively little of it deals with the non-tsetse transmitted trypanosome, *T. evansi*. The work described in this thesis set out to provide more information than is currently available on the immune responses to *T. evansi* in sheep and also to attempt to determine the extent of the immunopathological consequences of the infection and the possible underlying mechanisms. This immunological study was carried out by utilising the advantages offered by modern cell typing and separation techniques, the use of monoclonal antibodies and *in vitro* cell culture assays.

Basically, the studies on sheep have shown that infections with *T. evansi* cause marked alterations in the expression of different lymphocyte phenotypes in the blood and efferent lymph and suppression of the responses of animals to both heterologous vaccine antigens and homologous trypanosomal antigens. The work has achieved the following principle observations:

1) Significant increases in both percentages and numbers of circulating peripheral blood B lymphocyte subsets many of which express the CD5 antigen (i.e. there is increase in $CD5^+$ B cells).

2) Consequent significant reduction in subsets expressing the T cell associated molecules CD5, CD4, CD8 and $\gamma\delta$ TcR.

3) Infection apparently depresses serum antibody responses to the trypanosomes and suppresses the responses to *Pasteurella haemolytica* vaccine, causing reduced inflammatory responses, suppression of the neutrophilia seen in normal sheep and a reduction in serum antibody responses, particularly IgG1 levels; infection appears to have no effect on immunological memory since animals which selfcure respond to secondary vaccination at a similar level to normal, uninfected sheep.

4) The significant alterations in T cell subsets and B cells seem to play a role in the depression of serum antibody responses since in animals which selfcure, there is a greater reduction in $CD8^+$ than $CD4^+$ T cells with a resultant increase in CD4:CD8 ratio, less increases in circulating B cells and virtually no increase in $CD5^+$ B cells.

Furthermore, examination of the effects of infection on local responses was investigated by cannulation of the efferent duct of peripheral lymph nodes draining the site of *Pasteurella haemolytica* vaccine administration, and the following principal observations were made. *T. evansi* infection results in:

1) The appearance of $CD4^+CD8^+$ (double positive, DP) lymphocytes
2) Large increases in $CD5^+$ B cell populations
3) Consequent decreases in efferent T cell populations
4) Terminal (late) decreases in efferent B cells output.

In addition, *in vitro* functional assays established that active infection depressed the ability of peripheral blood lymphocytes to respond to specific stimulation with either *P. haemolytica* or trypanosomal antigens or to stimulation with either the T or B cell mitogens, Con A or LPS respectively. Moreover, *in vitro* functional studies utilising both cell depletion and separation assays identified $CD8^+$ cells and cells of the monocyte/macrophage series as playing key roles in trypanosome-induced immunodepression but showed that the monocyte plays the predominant role.

These observations suggest that trypanosomes induce generalised immunosuppression by a combination of immunological events originally intended for protection, but which eventually contribute to immunological defects. The early appearance of large numbers of $CD5^+$ B cells is probably directed towards increased production of natural IgM autoantibodies which usually act as first line of defence in infectious processes by enhancing phagocytosis and complement-mediated lysis (Casali and Notkins, 1989) and also by the enhancement of an ongoing specific immune response. Indeed, it has been shown that RF-like IgM antibodies derived from $CD5^+$ B cells completely protect rats from infection with *Trypanosoma lewisi* (Clarkson and Mellow, 1981). However, the autoantibodies produced by $CD5^+$ B cells are polyreactive, binding a variety of self and exogenous antigens with low affinity (Casali *et al.*, 1987; Casali and Notkins, 1989; Minoprio, 1991). The persistence and continued expansion of the $CD5^+$ B cell population as the infection progresses undermines clonal selection for the generation of antigen specific B cells producing enough VAT-specific, monoreactive, high affinity IgM and IgG antibodies capable of eliminating the parasites. This results in nonspecific B cell hyperplasia and IgM hypergammaglobulinaemia which is incapable of controlling the infection.

The failure to control B cell activation and the production of polyreactive IgM antibodies is likely to be related to the alterations in the proportions and numbers of $CD4^+$ and $CD8^+$ T cells in the blood and lymph of infected animals and the appearance of double positive T cells. Both CD4 and CD8 play a central role in

immune mechanisms and impart sensitivity and plasticity to immune responses. They enhance the avidity of T cell interaction with its APCs and promote TcR interaction with its appropriate antigen. In addition, these subsets are produced at certain optimal physiological ratios which enables them to interact and synthesise cytokines for effective and controlled cellular and humoral immunity against invading organisms. The roles of the $CD4^+$ and $CD8^+$ cells in the induction and regulation of sterile immunity dictates that adverse alterations in their production will determine the course and outcome of a disease process. Thus, in infected sheep, where there was a greater decrease in $CD4^+$ than $CD8^+$ cells, with a resulting decrease in CD4:CD8 ratio, it is likely that an enhanced IFN-γ production by the $CD8^+$ T cells would have inhibited $CD4^+$ T cell proliferation and provision of antigen-specific help to B cells.

Alterations in $CD4^+$ and $CD8^+$ cells leading to an upset in cytokine balance and failure of regulatory or stimulatory signals play a significant role in the induction of sterile immunity or mediation of aberrant immunological responses and have been demonstrated in both human and animal diseases. In the mouse for instance, where $CD4^+$ T cells have been subdivided on the basis of cytokine production into T_H1 and T_H2 subsets (Mosmann *et al.*, 1986), it has been shown that the predominance of one or the other of these subsets and their respective cytokines promotes resistance or susceptibility to *Leishmania major* infection (Bogdan *et al.*, 1990; Locksley and Scott, 1991). Recovery from infection by resistant mouse strains is closely associated with higher levels of T_H1 and production of IL-2 and IFN-γ. Increased numbers of T_H2 in other mouse strain is associated with high levels of IL-4 and susceptibility to *L. major* infection (reviewed in Bogdan *et al.*, 1990; Locksley and Scott, 1991).

In man, the progressive depletion of $CD4^+$ cells and expansion of the $CD8^+$ subset in subjects infected with HIV results in subsequent severe cellular and humoral immune dysfunction syndrome known as AIDS (Kornfeld *et al.*, 1982; Fahey, *et al.*, 1984; Lewis *et al.*, 1985; Melbye *et al.*, 1986; Cooper *et al.*, 1988). Furthermore, it has been shown experimentally, that *T. cruzi* in man mediates immunosuppression through the inhibition of CD4 and CD8 expression by activated T cells and by the suppression of both IL-2 production and IL-2 receptor expression by activated $CD4^+$ and $CD8^+$ cells (Sztein *et al.*, 1990). Moreover, activation of $CD8^+$ T cell phenotype (putative T suppressor cells) is implicated in the suppression of cellular immune responses to *Plasmodium falciparum* antigen during acute falciparum malaria (Riley *et al.*, 1988; 1989). In sheep, the depletion of the $CD4^+$ cells and increase in $CD8^+$ cells caused by respiratory syncytial virus (RSV) infection is accompanied by enhanced susceptibility to *P. haemolytica* infection. The

enhanced susceptibility is characterised by increased bacterial growth and mortality rates, with the alterations in lymphocyte phenotypes occurring 5-10 days after RSV infection. This coincides with the period of heightened susceptibility to superinfection with *P. haemolytica* (Sharma and Woldehiwet, 1990, 1991a, b; Sharma *et al.*, 1990).

Aberrations in the expression of CD4 and CD8 has also been noted in sheep infected with *Chlamydia psittaci* where early increased output of the subsets from lymph node draining the site of infection was followed by marked depletion in $CD4^+$ cells and a reduction in CD4:CD8 ratio from 3.5 to 1.7 (Huang *et al.*, 1991). In bovine *T. congolense* trypanosomiasis where changes in $CD4^+$ and $CD8^+$ cells have been reported (Ellis *et al.*, 1987; Williams, *et al.*, 1991), it has also been suggested that these changes probably underlie resistance or susceptibility of the host to the infection. For instance, in both primary and challenge infections of trypanosusceptible Boran cattle with *T. congolense*, there was persistence in the decreases in $CD4^+$ and $CD8^+$ cells in the blood with the animals requiring drug cure to stop death, whereas in the trypanotolerant N'dama, only the $CD8^+$ T cells decreased between days 7-21 p.i., after which they selfcured (Williams *et al.*, 1991).

The finding that monocyte removal substantially restores *in vitro* proliferative responsiveness to Con A while the removal of $CD8^+$ cells alone did not fully restore their responsiveness to specific antigen stimulation indicates the complexity of the immunosuppressive action of trypanosomes.

Based on the observed increase in $CD4^+CD8^+$ cells, the subsequent changes in $CD4^+$ and $CD8^+$ T cells, the B cell hyperplasia and increase in $CD5^+$ B cells, and the effects of the removal of the monocytes and $CD8^+$ cells on cell proliferative responses *in vitro*, it is possible to formulate a working hypothesis explaining the mechanisms responsible for trypanosome-induced immunosuppression. It is possible that trypanosomes interact initially with monocytes which are triggered to increased production of PGE_2 and release of IL-1. The released IL-1 potentiates PGE_2 synthesis by further activation of macrophages. PGE_2 directly inhibits IL-2 secretion by $CD4^+$ cells (Darji *et al.*, 1991b) and thus T cell proliferation and differentiation are inhibited. Moreover, the IL-1 produced by macrophages induces the T_H1 subset of $CD4^+$ T cells and $CD8^+$ cells to increased production of IFN-γ which in turn inhibits the expression of IL-2R on $CD4^+$ and $CD8^+$ T cells (Sileghem *et al.*, 1989a,. b; Darji *et al.*, 1991a, b). The inhibition of IL-2R expression prevents the binding and internalisation of IL-2 by T cells and a failure in the signal transduction required for activated lymphocytes to progress through their full cell proliferation and differentiation cycles. This results in failure of T cell function and may also explain the presence of double positive lymphocytes which are then

possibly destroyed by apoptosis with the consequent decrease in cell numbers. Furthermore, the failure in T cell differentiation and function will result in inability to regulate conventional and $CD5^+$ B cell hyperplasia and in the failure of the provision of antigen specific help to B cells for the production of sterilising amounts of antigen-specific antibodies.

Obviously, a lot more work will be needed to fully elucidate the exact role of the changes observed in this study and to validate the above hypothetical sequence of events which is based on the principal observations made in this study and on other published work on the subject. In other words the following line of future work might be contemplated:

1) Cell sorting of the $CD4^+$ and $CD8^+$ T cells from infected animals and assays of their cytokine production *in vitro*. This will not only shed some light on the effects of their altered expression on cytokine production but will highlight which subset of $CD4^+$ cells is being activated or suppressed.

2) Cell sorting of the $CD4^+CD8^+$ population and studying their functional behaviour, if any, *in vitro*, isolating their DNA and studying the pattern of their oligonucleosomal degradation fragments and the pattern of the rearrangement of their TcR genes in order to establish whether they represent a population dying by apoptosis or normal immature cells undergoing differentiation.

3) FACS sort the $CD5^+$ B cell population and study their antibody production patterns *in vitro*. This will establish whether they actually produce polyreactive low affinity rheumatoid factor-like autoantibodies in infected livestock.

4) Further *in vitro* lymphoproliferative studies on the role of macrophages through PGE_2 and H_2O_2 synthesis and release during *T. evansi* infections in ruminants by employing PG and H_2O_2 synthesis inhibitors such as indomethacin and catalase respectively.

Few scientific investigative studies have in the end provided answers to all the initial questions without succeeding in raising more questions than have actually been answered. It is clear from the various results and discussions that this thesis (is one of those studies that) has also managed to provide more questions than answers. It is hoped however that in addition to its moderate contribution to efforts geared towards unravelling the mechanisms underlying trypanosome-induced immunosuppression, it has been able to suggest clearly where next to look in search of answers to the enigmatic subject of trypanosome-induced immunopathology.

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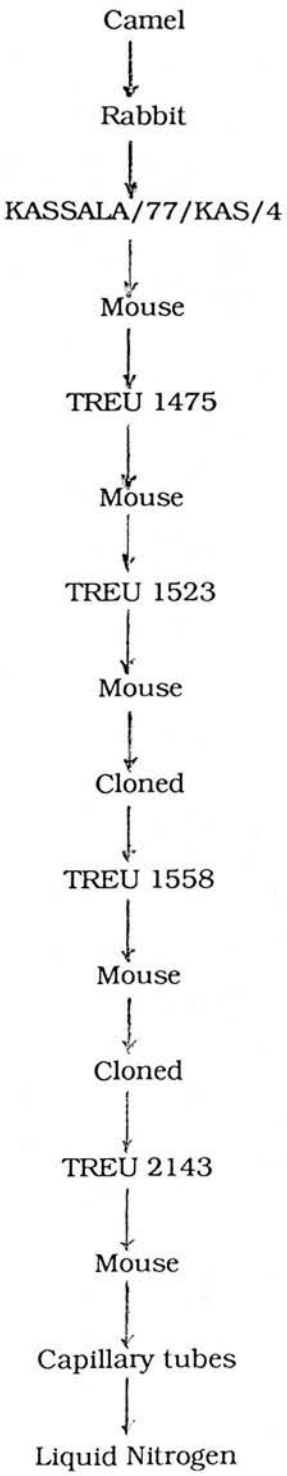
APPENDICES

Appendix I

Derivation of *T. evansi* TREU 2143

Trypanosoma evansi



Appendix II

Buffers and Reagents

1. **Phosphate Buffered Saline Glucose** (PSG, pH 8.0)

(a) Preparation of stock phosphate saline (PS stock)

Disodium hydrogen phosphate, anhydrous ($Na_2 HPO_4$)	-	14.38 g
Sodium dihydrogen phosphate ($NaH_2 PO_4.2H_20$)	-	0.78 g
Sodium chloride (NaCl)	-	4.25 g
Make up in warm distilled water to		1000 ml

(b) To prepare PS for use dilute 6 parts of PS stock with 4 parts of distilled water.

(c) Prepare PSG by adding glucose to give a 1 percent (1 gm per 100 ml) solution.

2. **Diethylaminoethyl Cellulose (DE-52)**

(a) Suspend 100 g DE-52 in 400 ml PSG

(b) Adjust pH to 8.0 with Molar orthophosphoric acid

(c) Wash four times with 400 mls PSG per wash

(d) Store overnight at 4^oC or freeze at -20^o until needed

3. **Immunofluorescence Buffer** (IFB)

Bovine serum albumin (Sigma)	1 %
Sodium azide	0.1%
Heparin	20.0 IU ml^{-1}

Make up in Phosphate Buffered Saline pH 7.2

4. **Paraformaldehyde Fixative**

(a) Preparation of Phosphate Buffered Saline (PBS, pH 7.2)

Sodium Chloride (NaCl)	-	8.00 g $litre^{-1}$
Posassium Chloride (kCl)	-	0.20 g $litre^{-1}$
Disodium hydrogen phosphate (Na_2HPO_4)	-	1.15 g $litre^{-1}$
Potassium dihydrogen phosphate (KH_2PO_4)	-	0.20 g $litre^{-1}$

Dissolve in 1000 ml distilled water

(b) Dissolve 1 gm paraformaldehyde in 50 ml warm PBS. Add 1M sodium hydroxide (NaOH) dropwise until the paraformaldehyde dissolves. Adjust pH to 7.2 and store at 4^oC.

5. **Tris-ammonium Chloride Buffer** (Tris-NH_4Cl)

(a)

0.17M Tris (hydroxymethyl) aminomethane	-	20.6 g
Distilled water	-	1000 ml

(b)

0.16M Ammonium Chloride	-	8.3 g
Distilled water	-	1000 ml

(c) To prepare lysing solution add 10 ml of (a) to 90 ml of (b) and adjust pH to 7.2 using 1M HCl.

6. **Carbonate/Bicarbonate Buffer** (pH 9.6)

Sodium carbonate	-	1.59 g
Sodium bicarbonate	-	2.93 g
Distilled water	-	1000 ml

7. **RPMI 1640 Complete Culture Medium**
RPMI 1640 supplemented with 2 mM L-glutamine, 100 $IUml^{-1}$ benzyl penicillin, 100 IU ml^{-1} Streptomycin, 5 x 10^{-5} M 2-mercaptoethanol, 2 g $litre^{-1}$ sodium bicarbonate and 10 percent heat inactivated fetal calf serum (FCS)

8. **RPMI 1640 Culture Wash Medium**
RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU ml^{-1} benzyl penicillin/streptomycin, 2 g $litre^{-1}$ $NaHCO_3$ and 1 percent FCS.

9. **Tris-HCl Buffer** (pH 8.4)
Dissolve 12.1 g Tris (hydroxy methyl) aminomethane in 100 ml distilled water to prepare a 1.0M stock solution. Titrate in a pH meter using 1M HCl to pH 8.4 and then dilute to the desired molarity with distilled water.

Appendix III

Protocols

1. **Anion Exchange Chromatography for the Separation of Bloodstream Trypanosomes**

(a) Collect infected mouse blood in a heparinised container.

(b) Pack equilibrated DE-52 in a mounted 20 ml syringe after partially plugging the outlet with sterile glass wool.

(c) Place a piece of sterile Whatman filter paper disc on top of the packed column and wash with 50 ml of sterile PSG.

(d) Place the trypanosome-containing blood on top of the column and elute it with PGS.

(e) Collect the trypanosome containing eluate in a sterile plastic universal bottle kept cool in an ice bath.

(f) Centrifuge at 2260 r.p.m. for 20 minutes at $4^{o}C$ and discard the supernatant.

(g) Resuspend the pelleted trypanosomes and wash three times in 25 ml sterile PSG.

(h) Count trypanosomes using an improved Neubauer Haemocytometer chamber and adjust the concentration to 10^{7} trypanosomes ml^{-1} of sterile PSG.

2. **Lymphoprep (Density gradient) Separation of Peripheral Blood Leucocytes**

(a) Draw 20 mls of jugular blood into sterile glass universal bottles containing sterile glass beads.

(b) Defibrinate the blood by gentle shaking for three minutes or until a discernible white fibrin clot forms.

(c) Remove fibrin clot and glass beads and centrifuge the blood at 1000 g for 20 minutes at $4^{o}C$.

(d) Carefully aspirate the buffy coat layer and mix it with an equal volume of PBS.

(e) Carefully layer over 8 mls of Lymphoprep (Nycomed) and centrifuge at 800 g for 30 minutes at $4^{o}C$.

(f) Using a sterile long-tipped Pasteur pipette recover the lymphocytes from the lymphoprep/plasma interphase.

(g) Wash cells three times using IFB or culture wash medium by centrifugation at 350 g for 10 minutes at $4^{o}C$.

(h) Resuspend cells in fresh buffer or medium, count and adjust to 10^{7} cells ml^{-1}.

3. **Tris-ammonium Chloride (Hypotonic lysis) Separation of PBLs**

(a) Prepare Tris-ammonium chloride as detailed in Appendix II(5).

(b) Collect 10 ml jugular blood into heparinised vacutainer (Becton-Dickinson).

(c) Mix blood with 35 ml prewarmed (37^{o}C) tris-ammonium chloride and allow to stand at room temperature for approximately three minutes for complete lysis of the red blood cells to occur.

(d) Centrifuge at 800 g for 15 minutes at 4^{o}C and discard supernatant.

(e) Resuspend cell pellet, wash three times, count and adjust cell concentration as above.

4. **ELISA Protocol**

I. Coat a 96 well ELISA plate with 100 µls of appropriately diluted *Pasteurella* or trypanosomal antigen and incubate overnight at 4^{o}C.

II. Discard contents and wash plates 3 times using PBS/Tween 20.

III. Dispense 100 µls of the appropriately diluted test and reference serum samples in triplicate wells or as required and incubate for 30 minutes at 37^{o}C.

IV. Wash three times in PBS/Tween 20.

V. For indirect ELISA, proceed to step VI. For double sandwich ELISA, dispense 100 mls of the appropriately diluted monoclonal antibodies as in III above, incubate for 30 minutes at 37^{o}C and wash three times in PBS/Tween 20.

VI. Using PBS/Tween 20, make appropriate dilutions of the horseradish peroxidate (HRP) conjugated anti-sheep antibody and dispense as in V above. Incubate for 30 minutes at 37^{o}C and wash as before.

VII. Dispense 100 mls of tetramethyl benzidine (TMB) in citrate buffer (enzyme substrate) to each well. Incubate at 37^{o}C for 15 minutes or leave in the dark at room temperature for colour to develop, usually 10-30 minutes. Stop enzyme action by adding 50 µls of 2M sulphuric acid. Read plate at 450 nm.

5. **Protocol for Lymphoproliferative Assay**

(a) Collect 20 mls of blood and separate the lymphocytes by density gradient centrifugation as described in Appenidx III(2).

(b) Count cells and adjust the concentration to 10^{7} cells ml^{-1} using RPMI 1640 complete culture medium (Appendix II(7)).

(c) Separate *T. evansi* as described in Appendix III(1).

(d) Make appropriate dilutions of the mitogens and antigens as appropriate using complete culture medium.

(e) Dispense the cells, mitogens and antigens as appropriate into 96-well flat-bottomed microculture plates at their respective optimal concentrations and make up the volume with complete culture medium to give a final volume of 200 µls per well.

(f) Incubate at 37^{o} in a humified atmosphere of 5 percent CO_2, 95 percent air for 72 hours.

(g) Pulse each well with 0.5 µCi of [^{3}H]-thymidine over the last 5 hours of incubation. Harvest cells onto glass filter paper discs using a semi-automated cell harvester (Multi Mash II) and oven-dry the filter paper at 60^{o}C over 30 minutes - 1 hour.

(h) With a thumb forceps, peel off the cell-containing discs from the filter paper and place each in a scintillation vial. Dispense 2 mls of scintillation fluid into each vial ensuring that the disc is well submerged.

(i) Determine DNA synthesis (thymidine uptake) by counting in a liquid scintillation beta-spectrometer.

Appendix IV

Addresses of Manufacturers

1. Amersham International p.l.c.
   Lincoln Place
   Green End
   Aylesbury
   Bucks HP20 2TP

2. BDH Ltd
   Broom Road
   Poole
   Dorset BH12 4NN

3. Becton-Dickinson
   Between Towns Road
   Cowley
   Oxford OX4 3LY

5. Difco Laboratories Ltd
   PO Box 14b
   Central Avenue
   East Molesey KT8 0SE

6. Dynatech Laboratories Ltd
   Daux Road
   Billingshurst
   West Sussex RH14 9SJ

7. Ethicon Ltd
   Edinburgh
   Scotland

8. Flow Laboratories Ltd
   Woodcock Hill
   Harefield Road
   Rickmansworth
   Herts WD3 1PQ

9. Gibco Ltd
   Unit 4
   Cowley Mill Trading Estate
   Longbridge Way
   Uxbridge UB8 2YG

10. Millipore UK Ltd
    11-15 Peterborough Road
    Harrow
    Middlesex HA1 2IH

11. MSE
    Sussex Manor Park
    Crawley
    West Sussex RH10 2QQ

12. Nordick Immunologicals
PO Box 544
Maidenhead
Berks SC6 2PW

13. Nycomed UK Ltd
Nycomed House
2111 Coventry Road
Sheldon
Birmingham B26 3EA

14. Scientific Supplies Co
Scientific House
Vine Hill
London EC1 5EB

15. Scottish Antibody Production Unit (SAPU)
Law Hospital
Carluke
Lanarkshire

16. Senono Bake Diagnostics
100 Cascade Drive
PO Box 2168 Allentown
Pennsylvania
USA

17. Serotec
22 Bankside Station Approach
Kidlington
Oxford OX5 1JE

18. Shandon Southern Products
Chadwick Road
Astmoor
Runcorn
Cheshire WA7 1PR

19. Sigma Chemical Co
3050 Spruce Street
PO Box 14508
St Louis
MO, USA 63178

20. Sterilin Ltd
Lampton House
Lampton Road
Hounslow
Middlesex TW3 4EE

21 The Binding Site Ltd
97 Vincent Drive
Edgbaston
Birmingham
B15 2SQ

22. Whatman Lab Sales Ltd
PO Box 6
Twyfords
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Berks RG10 9NL

Appendix V

Statistical analysis of peripheral blood data in *T. evansi* infected sheep

Although in Chapter 4, the results of the two sheep which selfcured and those of the six that remained parasitaemic were presented separately, the overall effect of *T. evansi* TREU 2143 infection on various clinical and blood parameters in the eight sheep was tested by comparing the preinfection data (day -7) with those of selected post infection days (i.e. the day of highest body temperature, before selfcure or drug treatment and after selfcure or drug treatment) using Student's paired t-test.

Temperature (^{o}C) : There was a significant increase in body temperature ($P<0.001$, day 15; $P<0.01$; day 40) following infection (Table 1).

Erythrocyte counts ($x10^6$ μl^{-1}): This decreased significantly ($P<0.01$; day 15) during the first two weeks of infection. Thereafter, although the count remained below preinfection values there was no significant difference between them ($P>0.05$) (Table 1).

Packed Cell Volume (%): There was a significant decrease in the PCV values following infection ($P<0.05$, day 15; $P<0.01$, day 40) (Table 1).

Total WBC Count ($x10^6$ ml^{-1}): This increased significantly by day 22 p.i. ($P<0.05$) and remained significantly high ($P<0.01$) four weeks after treatment.

Lymphocyte (%): Infection was characterised by significant lymphocytosis which persisted even four weeks after drug therapy ($P<0.001$) (Table 1).

Neutrophils and Eosinophils (%): A significant decrease ($P<0.001$) in neutrophils and eosinophils was observed which is a reflection of the lymphocytosis (Table 1).

Monocyte (%): Although an early increase was observed in the monocytes it was not significantly higher than preinfection values (Table 1).

Percent T cell subsets ($CD5^+$, $CD4^+$, $CD8^+$, $\gamma\delta^+$ T cells): Infection resulted in a highly significant decrease ($P<0.001$) in the proportions of various T cell subsets especially from day 22 post infection (Table 2).

Percent B. cells (sIg^+, $CD45R^+$, $CD1^+$, MHC II^{++} cells): A consequently highly significant increase in circulating B cells ($P<0.001$) was observed in all infected sheep (Table 2).

Days	N	Mean	STDEV	SE MEAN	T	P value
			1. Temperature (^{o}C)			
-7 Vs 15*	8	0.5000	0.2268	0.0802	6.24	0.0004
40*	8	0.3875	0.2696	0.0953	4.07	0.0049*
92	8	0.0625	0.1188	0.0420	1.49	0.1800
			2. Erythrocytes (x 10^{6} μl^{-1})			
-7 Vs 15*	8	1.688	1.305	0.462	3.66	0.0082*
40	8	0.000	1.437	0.508	0.00	1.000
92	8	0.125	0.868	0.307	0.41	0.7000
			3. Packed Cell Volume (%)			
-7 Vs 15*	8	4.500	4.408	1.558	2.89	0.0230*
40*	8	4.250	3.196	1.130	3.76	0.0072*
92	8	0.875	2.850	1.008	0.87	0.4100
			4. TWBC Counts (x 10^{6} ml^{-1})			
-7 Vs 15	8	-0.387	2.169	0.767	-0.51	0.6300
22*	8	2.738	2.629	0.929	2.95	0.0200*
40*	8	2.513	2.548	0.901	2.79	0.0270*
92*	8	1.950	1.761	0.622	3.13	0.0170*
			5. Lymphocytes (%)			
-7 Vs 15*	8	14.188	4.464	1.578	8.99	0.0000*
22*	8	14.475	5.047	1.785	8.11	0.0000*
40*	8	20.500	8.924	3.155	6.50	0.0003*
92*	8	9.438	5.716	2.021	4.67	0.0024*
			6. Neutrophils (%)			
-7 Vs 15*	8	10.625	4.479	1.679	6.33	0.0004*
22*	8	10.500	5.593	1.978	5.31	0.0012*
40*	8	16.125	8.835	3.124	5.16	0.0014*
92	8	7.625	7.918	2.799	2.72	0.029
			7. Eosinophils (%)			
-7 Vs 15*	8	3.437	1.635	0.578	5.95	0.0006*
22*	8	3.250	1.439	0.509	6.39	0.0004*
40*	8	3.250	1.648	0.582	5.58	0.0009*
92	8	1.750	2.563	0.906	1.93	0.0950
			8. Monocytes (%)			
-7 Vs 8	8	2.125	3.603	1.274	1.67	0.14
22	8	0.625	2.100	0.743	0.84	0.43
40	8	1.125	2.083	0.736	1.53	0.17
92	8	0.062	2.290	0.810	0.08	0.94
			9. $CD5^{+}$ T cells (%)			
-7 Vs 5*	8	8.750	10.403	3.678	2.38	0.0490*
15	8	4.125	7.120	2.517	1.64	0.1500
22*	8	15.125	5.842	2.065	7.32	0.0001*
40*	8	18.875	9.172	3.243	5.82	0.0007*
92	8	3.250	9.736	3.442	0.95	0.3800

Days	N	Mean	STDEV	SE MEAN	T	P value
			10. $\underline{CD4^+ T}$ cells (%)			
-7 Vs 5	8	2.125	5.540	1.959	1.08	0.3100
15	8	-0.125	4.155	1.469	-0.09	0.9300
22*	8	7.750	2.866	1.013	7.65	0.0001*
40*	8	13.250	4.301	1.521	8.71	0.0000*
92	8	5.250	9.130	3.228	1.63	0.1500
			11. $\underline{CD8^+ T}$ cells (%)			
-7 Vs 5*	8	3.000	3.024	1.069	2.81	0.026*
15	8	0.625	4.955	1.752	0.36	0.73
22*	8	7.375	1.685	0.596	12.38	0.0000*
40*	8	9.125	2.997	1.060	8.61	0.0000*
92*	8	3.500	3.665	1.296	2.70	0.030*
			12. $\underline{\gamma\delta\ T\ cells}$ (%)			
-7 Vs 5*	8	4.250	2.188	0.773	5.49	0.0010*
15*	8	10.750	3.196	1.130	9.51	0.0000*
22*	8	6.375	2.925	1.034	6.17	0.0005*
40*	8	6.750	2.866	1.013	6.66	0.0003*
92*	8	6.000	3.207	1.134	5.29	0.0012*
			13. $\underline{sIg^+ cells}$ (%)			
-7 Vs 15	8	2.875	12.206	4.315	0.67	0.5300
22*	8	15.250	11.548	4.083	3.74	0.0074*
40*	8	20.750	9.852	3.483	5.96	0.0006*
92	8	4.500	7.368	2.605	1.73	0.1300
			14. $\underline{CD45R^+ cells}$ (%)			
-7 Vs 15	8	9.250	13.145	4.647	1.99	0.0870
22*	8	15.875	12.334	4.361	3.64	0.0084*
40*	8	28.750	8.225	2.908	9.89	0.0000*
92	8	4.250	12.714	4.495	0.95	0.3800
			15. $\underline{CD1^+ cells}$ (%)			
-7 Vs 15	8	6.125	9.493	3.356	1.82	0.1100
22*	8	7.125	4.422	1.563	4.56	0.0028*
40*	8	16.750	9.254	3.272	5.12	0.0015*
			16. $\underline{MHC\ II^+ cells}$ (%)			
-7 Vs 15	8	4.000	11.208	3.991	1.00	0.3500
22*	8	19.000	8.816	3.117	6.10	0.0005*
40*	8	27.125	6.621	2.341	11.59	0.0000*
92	8	4.500	9.636	3.407	1.32	0.2300

* Indicates days when there were significant differences between pre- and post-infection values.

PUBLICATIONS

Some aspects of this work were presented in scientific meetings and published as follows:

1. ONAH, D.N., HOPKINS, J. and LUCKINS, A.G. (1990).
Lymphocyte phenotype kinetics during *Trypanosoma evansi* infection in sheep. Royal Society of Tropical Medicine and Hygiene, Scottish Branch, Laboratory Meeting, Edinburgh, 8 May 1990. Published in: *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84:** 895.

2. ONAH, D.N., HOPKINS, J. and LUCKINS, A.G. (1991).
Infection with *Trypanosoma evansi* alters expression of lymphocyte surface antigens and suppresses response to vaccination with *Pasteurella haemolytica*. Instituut voor Tropische Geneeskunde "Prins Leopold" Institut de Medecine Tropical "Prince Leopold" and The British Society for Parasitology. International Colloquim: Trypanosomiasis Seminar, 11-13 December 1991, Antwerp, Belgium.

Transactions of the Royal Society of Tropical Medicine and Hygiene (1990) 84, 892–896

Royal Society of Tropical Medicine and Hygiene

Scottish Branch
Laboratory Meeting, Edinburgh, 8 May 1990

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Lymphocyte phenotype kinetics during *Trypanosoma evansi* infection in sheep

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The cellular immune response of sheep to experimental trypanosomiasis was studied using a panel of anti-sheep lymphocyte monoclonal antibodies. Sheep were infected by intravenous injection of 10^5 bloodstream forms of *Trypanosoma evansi* (TREU 2143). Peripheral blood leucocytes were separated from jugular vein blood twice weekly using Tris-ammonium chloride lysis and changes in lymphocyte phenotypic expression analysed by flow cytometry. There was a persistant lymphocytic leucocytosis from day 22 after infection up to and including the fourth week. This was due to a B-cell lymphocytosis as evidenced by the concurrent increases in the percentage of cells expressing the leucocyte common antigen (LCA P.220), class II major histocompatibility complex (MHC) antigen and surface immunoglobulin (SIg) antigen. These increases were paralleled by decreases in the percentages of T-cell subsets, namely CD-5+, CD-4+, CD-8+ and SBU-T19+ cells, and were especially marked from day 22 after infection. There were intermittent increases in the CD-4/CD-8 cell ratio which resulted from greater decreases in the percentage of CD-8 expression.

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INFECTION WITH *TRYPANOSOMA EVANSI* ALTERS EXPRESSION OF LYMPHOCYTE SURFACE ANTIGENS AND SUPPRESSES RESPONSE TO VACCINATION WITH *PASTEURELLA HAEMOLYTICA*

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In sheep infected with *Trypanosoma evansi* there are marked changes in different lymphocyte sub-populations in the peripheral blood during the course of infection. The number of B cells increases from 22 days after infection and there is a decrease in the number of lymphocytes expressing CD4 and CD8 antigens. The proportion of B cells expressing CD5 antigen increases from a normal level of approximately 5% to over 90% early in infection. These changes persist until the animal is treated with a trypanocidal drug, when there is a gradual return to pre-infection values. In addition, the changes are also associated with immunosuppression, for example, the humoral responses to both primary and secondary immunization with *Pasteurella haemolytica* vaccine is severely depressed in *T. evansi*-infected animals.

Analysis of lymphocyte populations draining from lymph nodes of sheep immunized with *P. haemolytica* has shown differences in response between infected and uninfected individuals. In uninfected animals there is an increase in CD5+ and CD4+ T cells commencing 3—4 days after immunization. B cells also show an increase but CD8+ lymphocytes show little alteration and the CD4/CD8 ratio remains high. In contrast, in infected sheep, the number of CD5+ and CD4+ cells is decreased compared to uninfected animals and there is no increase in B cells following immunization. The majority of B cells express CD5 antigen. There is an increase in immature T cells expressing both the CD4 and CD8 antigens simultaneously. These alterations in lymphocyte populations correlate with the differences in response to *P. haemolytica* seen between infected and uninfected sheep and it is possible therefore that the changes in these cell populations might be responsible for the impaired immune responses seen in *T. evansi* infected animals.